STEM CELLS TRANSLATIONAL MEDICINE®

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM (iPS) CELLS

Neural Progenitors Derived From Human Induced Pluripotent Stem Cells Survive and Differentiate Upon Transplantation Into a Rat Model of Amyotrophic Lateral Sclerosis

IULIANA RISTEA POPESCU,[a] CHARLES NICAISE,[a] SONG LIU,[b] GRÉGOIRE BISCH,[c] SARAH KNIPPENBERG,[d] VALERY DAUBIE,[a] DELPHINE BOHL,[c,*] ROLAND POCHET[a,*]

Key Words. Spinal cord injury • Stem cell transplantation • Rat model • Neurons • Neural stem cell • Neural differentiation

[a]Laboratory of Histology, Neuroanatomy & Neuropathology, Université Libre de Bruxelles, Brussels, Belgium; [b]UMR 788, INSERM et Université Paris-Sud, Le Kremlin-Bicêtre, France; [c]Retrovirus and Cell Transfer Unit, Institut Pasteur, INSERM U622, Paris, France; [d]Department of Neurology Hannover Medical School, Centre for Systems Neuroscience, Hannover, Germany

[*]Contributed equally.

Correspondence: Roland Pochet, Ph.D., Laboratory of Histology, Neuroanatomy & Neuropathology, Université Libre de Bruxelles, 808 Route de Lennik, B-1070 Brussels, Belgium. Telephone: 32-25556374; Fax: +32-25556285; E-Mail: rpochet@ulb.ac.be

Received April 21, 2012; accepted for publication December 6, 2012; first published online in SCTM *EXPRESS* February 14, 2013.

1066-5099/2013/$20.00/0

http://dx.doi.org/10.5966/sctm.2012-0042

ABSTRACT

Human induced pluripotent stem cells (iPSCs) offer hope for personalized regenerative cell therapy in amyotrophic lateral sclerosis (ALS). We analyzed the fate of human iPSC-derived neural progenitors transplanted into the spinal cord of wild-type and transgenic rats carrying a human mutated SOD1(G93A) gene. The aim was to follow survival and differentiation of human neural progenitors until day 60 post-transplantation in two different in vivo environments, one being ALS-like. iPSC-derived neural progenitors efficiently engrafted in the adult spinal cord and survived at high numbers. Different neural progenitor, astroglial, and neuronal markers indicated that, over time, the transplanted nestin-positive cells differentiated into cells displaying a neuronal phenotype in both wild-type and transgenic SOD1 rats. Although a transient microglial phenotype was detected at day 15, astroglial staining was negative in engrafted cells from day 1 to day 60. At day 30, differentiation toward a neuronal phenotype was identified, which was further established at day 60 by the expression of the neuronal marker MAP2. A specification process into motoneuron-like structures was evidenced in the ventral horns in both wild-type and SOD1 rats. Our results demonstrate proof-of-principle of survival and differentiation of human iPSC-derived neural progenitors in in vivo ALS environment, offering perspectives for the use of iPSC-based therapy in ALS. STEM CELLS TRANSLATIONAL MEDICINE *2013;2:167–174*

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by degeneration and death of cortical and spinal motor neurons, leading to muscle atrophy, paralysis, and finally death by failure of the respiratory muscles. This occurs 3–5 years postdiagnosis [1] and, except Riluzole (Sanofi-Aventis, Diegem, Belgium, http://www.sanofi.be), which prolongs survival by a few months [2], no treatment is currently available [3]. This lack is principally due to the poor understanding of the etiology of motor neuron degeneration in ALS. Only approximately 10% of ALS cases are inherited and caused by dominant (rarely recessive) autosomal mutations in several genes [3], of which those of the free radical scavenging enzyme Cu/Zn superoxide dismutase 1 (SOD1) accounts for 20% [4]. Despite our improved understanding of ALS pathogenesis from the use of transgenic animal models, there are still no effective treatments or preventive strategies in humans, and ALS remains an incurable orphan disease [5]. Stem-cell-based therapies have emerged as a potential solution in many neurodegenerative diseases, including ALS [6]. The transplantation of stem cell-derived neural progenitors may have a beneficial effect: not only can they replace motor neurons already lost but they can also serve as source of neurotrophic factors and modifiers of the inflammatory environment, thus counteracting degeneration and death of motor neurons. Spinal motor neurons have been successfully generated (in vitro and in vivo, both in rodent and humans) from various stem cell sources such as embryonic stem cells and neural stem cells (NSCs) (reviewed in [7]). Studies have also evaluated the therapeutic potential of bone marrow-derived-human mesenchymal stem cells and human umbilical cord blood cells, but modest or no therapeutic benefit was obtained when they were transplanted in ALS patients, with safety and efficacy concerns (reviewed in [6]). In a previous work we have started such a study by using intravenously injected rat NSCs into SOD1 rats [8]. Although these cells undergo a massive apoptosis after a few days

of injection, several survived, crossed the blood-brain barrier, differentiated, and engrafted into the spinal cord of SOD1 rats.

Induced pluripotent stem cells (iPSCs) can be easily generated from patient's fibroblasts and further differentiated in vitro in specific cell types, including neural precursors, neurons, and motor neurons [9–14]. Few studies have described the generation of iPSCs from ALS patients and their differentiation into motor neurons for ALS disease modeling [10, 13, 15, 16]. Also, no report has described yet the in vivo fate of transplanted iPSCs into an ALS-like environment. Here we describe the intraparenchymal transplantation of human iPSC-derived neural progenitors (iPSC-NPs) into an ALS environment and characterize their cellular fate in vivo. Results indicate that human iPSC-NPs successfully survived into both wild-type and ALS-like environments, localized in the gray matter, and progressively differentiated into human mature neurons, some of them having motoneuronal morphologies.

Materials and Methods

Animals

Surgical procedures performed on animals were approved by the institutional boards for the care and use of experimental animals of the Université Libre de Bruxelles. Wild-type (WT) and transgenic SOD1(G93A) rats bred on the Sprague-Dawley genetic background (002148-T, NTac:SDTg (SOD1G93A)L26H) carrying the mutant human SOD1 gene were obtained from Taconic Farms Europe (Denmark, http://www.taconic.com). They were housed in our conventional animal facilities, maintained in a 12-hour light/12-hour dark cycle, with ad libitum access to food and water, in accordance with the Animal Welfare Guidelines of the Université Libre de Bruxelles (http://www.ulb.ac.be/facs/medecine) and with European Community Council Directives (86/609/EEC and 87-848/EEC).

Preparation of Human iPSC-NPs for Transplantation

Skin fibroblasts were obtained from the Centre de Ressources Biologiques in Lyon, France, with the approval of competent authorities. A statement of biological samples was made according to French laws formulated by the Ministère de la Recherche and to the Comité de Protection des Personnes, Ile de France (DC 2009-1067). An export authorization was also obtained to send cells to Belgium for cell transplantation. iPSCs and iPSC-NPs were obtained as previously detailed [12, 17]. Briefly, iPSCs were generated following forced expression of OCT4, SOX2, KLF4, and c-MYC transcription factors with retroviral vectors, and were fully characterized as pluripotent stem cells [17]. iPSCs were maintained on irradiated mouse embryonic fibroblast feeder layers in the following medium (iPSC medium): Dulbecco's modified Eagle's medium/F12 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) containing 20% Knock-Out Serum Replacement (Invitrogen), 10 ng/ml fibroblast growth factor 2 (FGF2) (Miltenyi Biotec, Paris, France, http://www.miltenyibiotec.com), 100 μM nonessential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Invitrogen), 50 U/ml penicillin, and 50 mg/ml streptomycin. Cultures were passaged every 5–10 days either manually or enzymatically with collagenase type IV (1 mg/ml; Invitrogen). For neural differentiation, iPSCs were collected as small clusters and resuspended in iPSC medium without FGF2. After 2 weeks, floating clusters were dissociated into single cell suspension with Accumax (PAA Laboratories, Linz, Austria, http://www.paa.at), and cell count and viability were determined with trypan blue. After centrifugation (5 minutes at 900 rpm), the medium was removed and cells were suspended in fresh medium at a concentration of 10^5 cells per microliter, prior to transplantation. Cell suspension prepared for injection (2 μl, corresponding to 200,000 cells) was kept on ice along the entire transplantation procedure and cell viability was assessed at the end of the session. The in vitro differentiation fate of the same iPSC-NP preparation was also assessed following dissociation and adhesion of single-cell suspension. As previously reported [17], 1 day after adhesion, all iPSC-NPs expressed the early neural progenitor marker nestin. Undifferentiated iPSCs labeled with an anti-OCT4 antibody were not detected in these cultures (data not shown).

Experimental Design

Presymptomatic (3-month-old) SOD1 and age-matched wild-type rats, under deep anesthesia induced by a combination of ketamine (60 mg/kg) and xylazine (7.5 mg/kg), were transplanted with human iPSC-NPs by injection of 10^5 cells per microliter, bilaterally targeting the ventral horns of the lumbar spinal cord (level L4–L5), after fixation of the spine in a stereotactic frame and laminectomies. Cells were delivered at a rate of 0.5 μl/minute by using a Hamilton syringe connected to a pump controller. The needle was inserted to a depth of 2.5 mm below the dorsal spinal cord surface and left in place for 5 minutes after each injection. The injection site was marked by a 9-0 nylon microsuture. After the operation, the incision was sutured with 4-0 nylon sutures. Rats were immunosuppressed with cyclosporine A (Neoral; Novartis International, Basel, Switzerland, http://www.novartis.com) at 10 mg/kg per day, added in drinking water. The immunosuppression started 5 days before transplantation and continued until the sacrifice. Rats (wild-type and SOD1) were sacrificed at days 15, 30, and 60 after transplantation. Animals were deeply anesthetized with the ketamine/xylazine solution as mentioned above and perfused intracardially with 4% paraformaldehyde. Lumbar spinal cord was removed and fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline and then washed with tap water and kept in 70% alcohol until paraffin embedding (Tissue-Tek; Sakura Belgium, http://www.sakuraeu.com).

Tissue Treatment and Immunostaining Procedures

Paraffin-embedded tissues were sectioned on a classic microtome (10 μm thickness), transversal sections collected on Superfrost Plus coverslips (ThermoFisher, http://www.be.fishersci.com) pretreated with poly-L-lysine.

Random spinal cord sections were colored with hematoxylin-eosin for injection site localization. For immununostaining, spinal cord sections with a visible injection site were dewaxed, rehydrated, permeabilized in Tris-buffered saline (TBS), and blocked with 10% normal goat serum for 1 hour at room temperature, preceded by microwave antigen retrieval in 10 mM citrate buffer. Sections were incubated with the primary antibody diluted in TBS with 1% serum, overnight at 4°C. In double immunofluorescence staining, the following primary antibodies were used: mouse anti-human mitochondria (MAB1273; Millipore, Billerica, MA, http://www.millipore.com), 1:20; rabbit anti-nestin (AB5922; Millipore), 1:150; mouse anti-glial fibrillary acidic protein (anti-GFAP) (GA5; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 1:100; rabbit anti-MAP2 [18], 1:100; rabbit

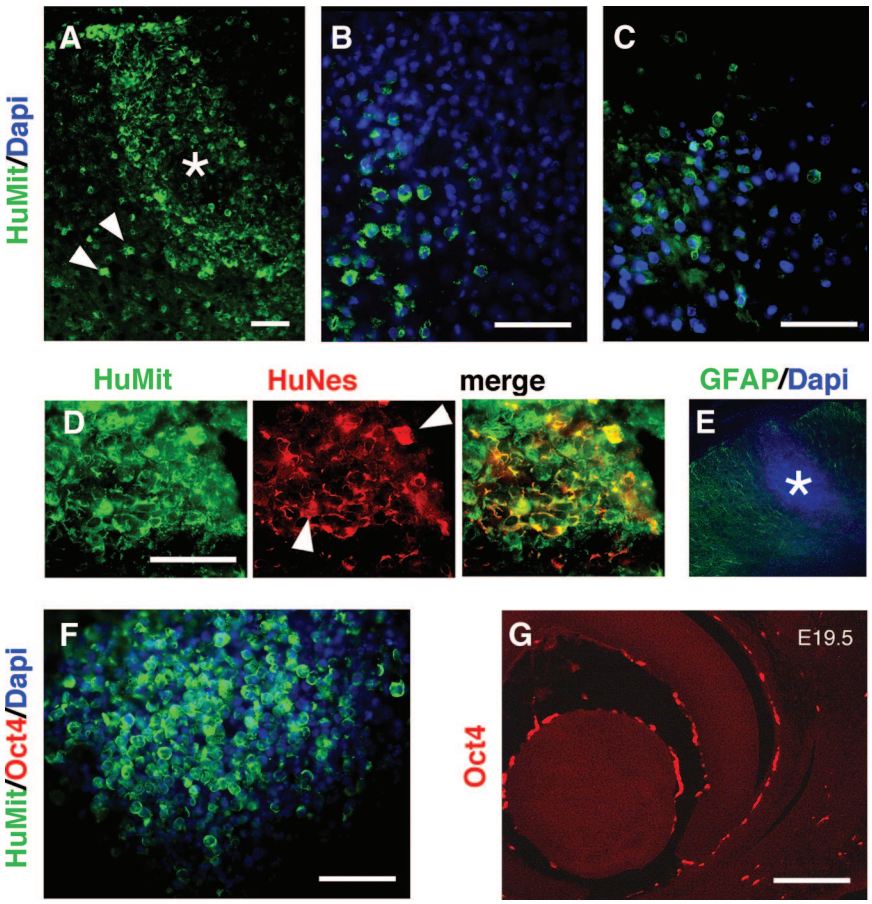

Figure 1. Human induced pluripotent stem cell-derived neural progenitors engraft after transplantation in the rat spinal cord (days 1–5). **(A):** Graft view in a transversal section of rat spinal cord 1 day after transplantation. Asterisk denotes the injection point. Injected progenitors were detected by immunolabeling against HuMit (shown in green; white arrows). **(B, C):** Representative images of cells engrafted in both wild-type ($n = 5$) **(B)** and SOD1(G93A) ($n = 5$) **(C)** spinal cords. Nuclei were counterstained with Dapi. **(D):** Representative image of neural progenitors coimmunostained against human Nestin (in red) and HuMit (in green). **(E):** Astroglyosis development around the graft. The spinal cord slice was labeled against the astrocyte marker GFAP (in green). Asterisk denotes grafted cells. **(F):** A transversal section of rat spinal cord labeled against Oct4 (in red) and HuMit (in green); nuclei were counterstained with Dapi (in blue). **(G):** Mouse embryonic nervous tissue at E19.5 labeled against Oct4 (in red) used as positive control for Oct4. Scale bars = 100 μm. Abbreviations: Dapi, 4′,6-diamidino-2-phenylindole; E, embryonic day; GFAP, glial fibrillary acidic protein; HuMit, human mitochondria; HuNes, human Nestin.

anti-Iba1 (Wako Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english), 1:100; rabbit anti-doublecortin (Abcam, Cambridge, MA, http://www.abcam.com), 1:100; rabbit anti-Ki67 (Millipore), 1:100; rabbit anti-Oct4 (Abcam), 1:100; and rabbit anti-α-synuclein (Abcam, Cambridge, MA, http://www.abcam.com), 1:100. Anti-human mitochondria were detected using a biotinylated goat anti-mouse IgG (1:200; Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) followed by incubation with streptavidin conjugated to Alexa 488 (1:100; Molecular Probes, Eugene, OR, http://probes.invitrogen.com). The secondary antibodies goat anti-rabbit conjugated to Alexa 594 (1:100; Invitrogen) and goat anti-mouse conjugated to Alexa 488 (1:100; Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com) were added for 1 hour at room temperature. In all experiments, negative controls were obtained by omitting the primary antibody. Sections were examined using the Axioplan microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Results

The feasibility of the surgical procedure was assessed by injecting human iPSC-NPs in one wild-type and one SOD1 rat, which were sacrificed 1 day post-transplantation. Human iPSC-NPs were identified with an antibody against human mitochondria (HuMit) (Fig. 1A, 1B). At day 1, cells were found in a compact mass at the injection site but also spread at a distance between 100 and 500 μm from the injection site (Fig. 1A). Numerous round cells, HuMit-positive, were identified in the spinal cord of both WT and SOD1 rat (Fig. 1B, 1C). As expected, the vast majority of transplanted cells expressed the neuronal progenitor marker nestin (Fig. 1D), showing that at this time point, the majority of neural progenitors were still immature. Because residual expression of exogenous genes used for reprogramming can block the differentiation of iPSCs into mature neuronal/glial types and reactivate pathways of oncogenesis [19], we analyzed the expression of Oct4, a marker of undifferentiated iPSCs, in transplanted cells. No Oct4 immunoreactivity was detected in the transplanted cells at day 1 (Fig. 1F, 1G), consistent with in vitro results (data not shown). The transplantation induced a moderate astrogliosis around the graft, but no GFAP-positive cells were detected within the graft (Fig. 1E).

The fate and survival of iPSC-NPs within an ALS environment were analyzed in 3-month-old SOD1 rats. We used age-matched wild-type animals as controls. Rats were sacrificed at days 15, 30, and 60 post-transplantation. At day 15, in both WT ($n = 5$) and SOD1 animals ($n = 4$), grafts contained numerous HuMit-positive cells, which also displayed a positive immunoreactivity for nestin. Figure 2 shows in the SOD1 animals fewer HuMit-positive cells expressing nestin at day 15 and 30, suggesting that between day 1 and day 15, nestin-positive neural progenitors differentiated more rapidly in the ALS environment. Cells were also stained with antibodies against the microglial and astrocytic markers Iba1 and GFAP. At day 15, a large proportion of transplanted cells expressed the microglial marker Iba1, in both WT and SOD1 animals, whereas at day 30, their amount significantly decreased in both groups of rats (Fig. 2A, 2B), suggesting a modification of iPSC-NP differentiation fate. We did not detect HuMit-positive cells expressing GFAP at any time point, in WT and

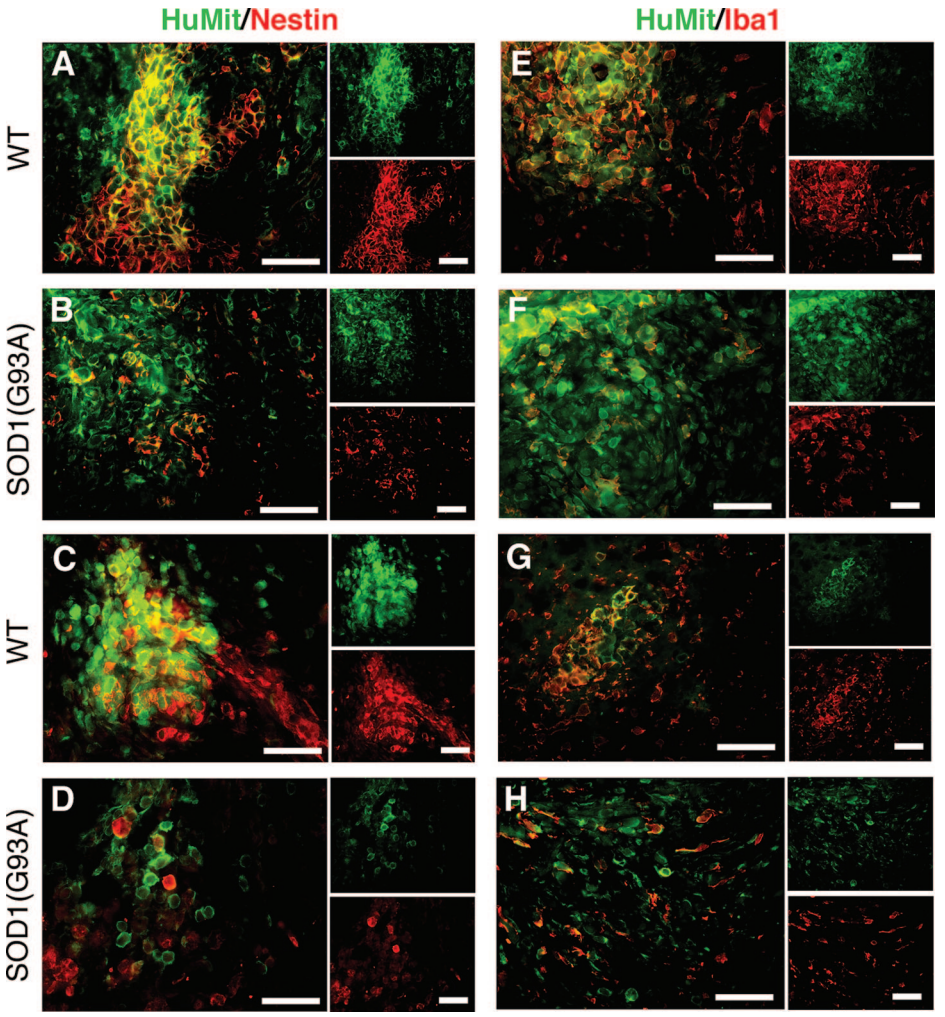

Figure 2. Induced pluripotent stem cell-derived neural progenitors (iPSC-NPs) at days 15 and 30 post-transplantation express Nestin and Iba1. Representative pictures of engrafted cells in WT and SOD1(G93A) rat spinal cords 15 days **(A, B, E, F)** and 30 days **(C, D, G, H)** post-transplantation. iPSC-NPs were identified with anti-HuMit antibody (in green) and colabeled against human Nestin (in red) or Iba1 (in red). For day 15: $n = 5$ (WT), $n = 4$ (SOD1(G93A)). For day 30: $n = 3$ (WT), $n = 4$ (SOD1(G93A)). Scale bars = 100 μm. Abbreviations: HuMit, human mitochondria; WT, wild-type.

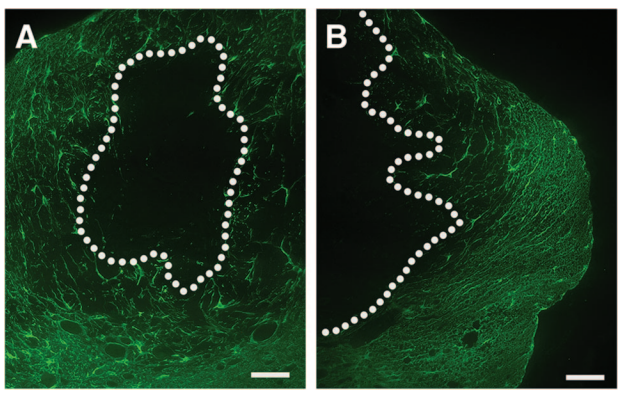

Figure 3. Lack of astrocyte differentiation in the graft at day 30 post-transplantation. Shown are representative images of wild-type (WT) and SOD1(G93A) rat spinal cords labeled against the astrocyte marker glial fibrillary acidic protein (in green). Dashed perimeter denotes graft border. $n = 3$ (WT), $n = 4$ (SOD1(G93A)). Scale bars = 100 μm.

SOD1 rats, suggesting that the differentiation process was not directed toward astrocyte formation (Fig. 3). For their potential for differentiation into oligodendrocytes, iPSC-derived neural progenitors were analyzed both in vitro and in vivo. In vitro, as described in the paper by Lemonnier et al. [17], O4- or NG2-positive cells were not detected in differentiated cultures 5 weeks after induction of differentiation. In vivo labelings with the anti-O4 antibody were also negative on spinal cord sections stained 30 and 60 days after transplantation.

In order to evaluate the long-term differentiation potential of transplanted cells into neurons, spinal cord sections were stained at days 30 and 60 with antibodies for the neuronal precursor doublecortin (DCX) and for the more mature neuronal marker MAP2 (Fig. 4). At day 30, we detected HuMit-positive cells expressing DCX in the spinal cord of both WT and SOD1 rats, and in addition, some HuMit-positive cells expressed the neuronal marker MAP2. Interestingly, some HuMit-positive cells expressing MAP2 displayed large shapes compared with the initially transplanted iPSC-NPs, reminiscent of the morphology of motor neurons (Fig. 4A). However, the majority of HuMit-positive cells were not stained for MAP2

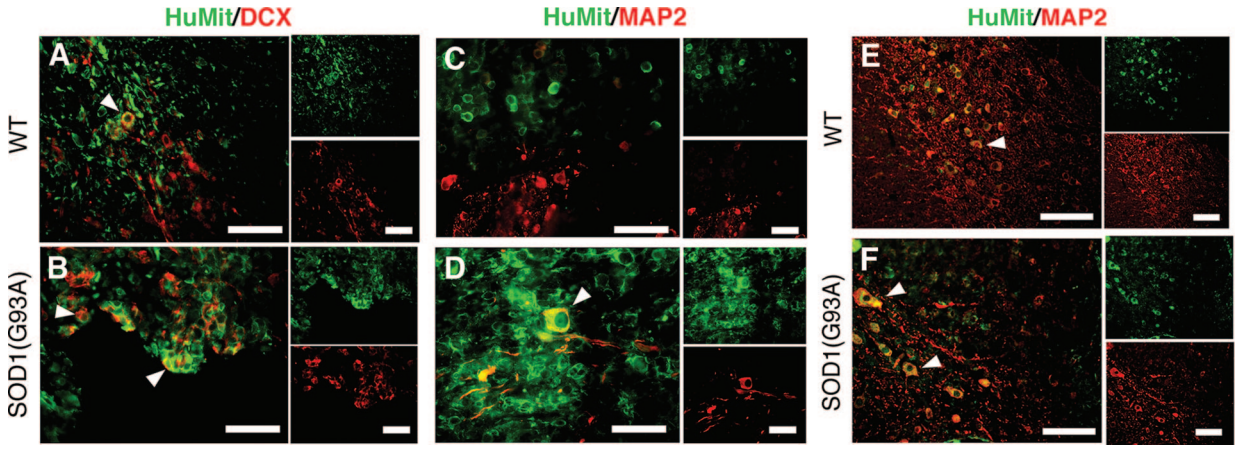

Figure 4. Induced pluripotent stem cell-derived neural progenitors differentiate into mature neurons at 30 and 60 days post-transplantation (arrowheads). **(A, B):** Representative pictures of engrafted cells in WT and SOD1(G93A) rat spinal cords, costained with anti-HuMit (in green) and anti-DCX (in red), at day 30 post-transplantation. **(C, D):** Representative pictures of engrafted cells in WT and SOD1(G93A) rat spinal cords, costained with anti-HuMit (in green) and anti-MAP2 (in red), at day 30 post-transplantation. Few MAP2-positive progenitors (**[D]**, arrowhead) with motor neuron morphology were detected. $n = 3$ (WT), $n = 4$ (SOD1(G93A)). **(E, F):** Numerous MAP2-positive cells (in red) were detected at day 60 post-transplantation in both WT and SOD1(G93A) rats (white arrowheads). Cells were identified with the anti-HuMit antibody (in green). $n = 2$ (WT), $n = 2$ (SOD1(G93A)). Scale bars = 100 μm. Abbreviations: DCX, doublecortin; HuMit, human mitochondria; WT, wild-type.

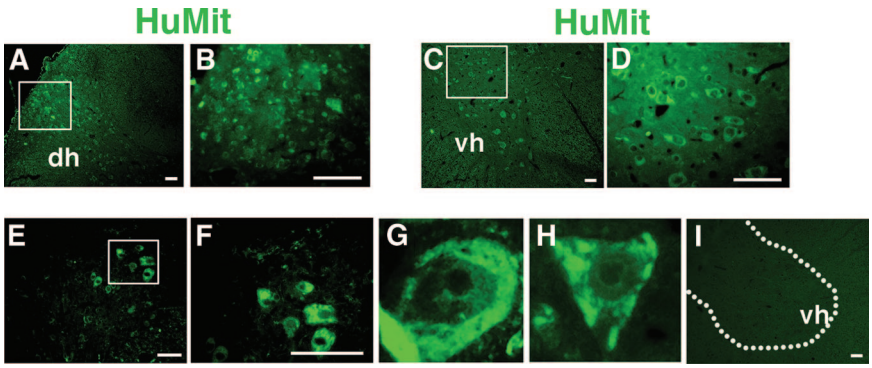

Figure 5. Morphological specification of induced pluripotent stem cell-derived neural progenitors at day 60 post-transplantation. **(A, B):** Representative pictures at different magnifications of transversal wild-type (WT) rat spinal cord sections immunostained for HuMit (in green) at the level of dorsal horn. **(C, D):** Representative pictures with different magnification of transversal WT rat spinal cord sections immunostained for HuMit (in green) at the level of ventral horn. **(E, F):** Representative pictures of transversal SOD1(G93A) rat spinal cord section immunostained for HuMit (in green) at the level of ventral horn. **(G, H):** HuMit-positive-progenitors with motoneuron-like morphology identified in the ventral horns of transplanted rats, versus HuMit-negative staining of the spinal cord in a nontransplanted WT rat **(I)**. In **(I)**, the dashed perimeter denotes the ventral horn border. Note the presence of the nucleolus inside the giant nucleus. $n = 2$ (WT), $n = 2$ (SOD1(G93A)). Scale bars = 100 μm. Abbreviations: dh, dorsal horn; HuMit, human mitochondria; vh, ventral horn.

at this stage, indicating that transplanted iPSC-NPs were not fully differentiated into a mature neuronal phenotype at day 30. Neuronal maturation was observed at day 60 post-transplantation, in both WT and SOD1 rats, as the majority of transplanted cells were MAP2-positive and spread in the gray matter of the spinal cord (Fig. 4B). Attempts to immunodetect the SMI32 and HB9 motoneuron markers in our model were unsuccessful because of technical problems. Nevertheless the morphology and the size of some HuMit-MAP2-positive neurons suggest that some iPSC-derived neurons were motor neurons (Fig. 4C–4F). We also observed that engrafted cells changed their morphology depending on their localization within the horn (Fig. 5A–5D), from the injection site toward ventral horns, where they acquired a motoneuron morphology (Fig. 5E–5H).

We also analyzed whether human iPSC-NPs degenerated in the WT or the ALS environment at days 30 and 60 post-transplantation. In one of four SOD1 rats sacrificed at day 30, we observed a tissue loss from the central area of the graft (Fig. 6A). To verify whether this could be due to degeneration of transplanted neural progenitors in the ALS environment, we analyzed whether the engrafted cells expressed markers of inclusion body formation and degeneration. No α-synuclein-positive or ubiquitin-positive (data not shown) cells were detected in the grafts. Only some α-synuclein-positive cells negative for HuMit were observed in the sample of tissue damage of the graft, indicating that tissue loss was not related to iPSC-NP degeneration in the ALS environment. Rare and transient Ki67-positive cells were detected in graft sections on day 30 in two animals (Fig. 6B, 6C).

Discussion

Induced pluripotent stem cells derived from patients with neurodegenerative diseases, which can give rise to lineages carrying the disease imprint, have been used to study disease-related phenotypes in vitro [20, 21]. This is a promising approach for

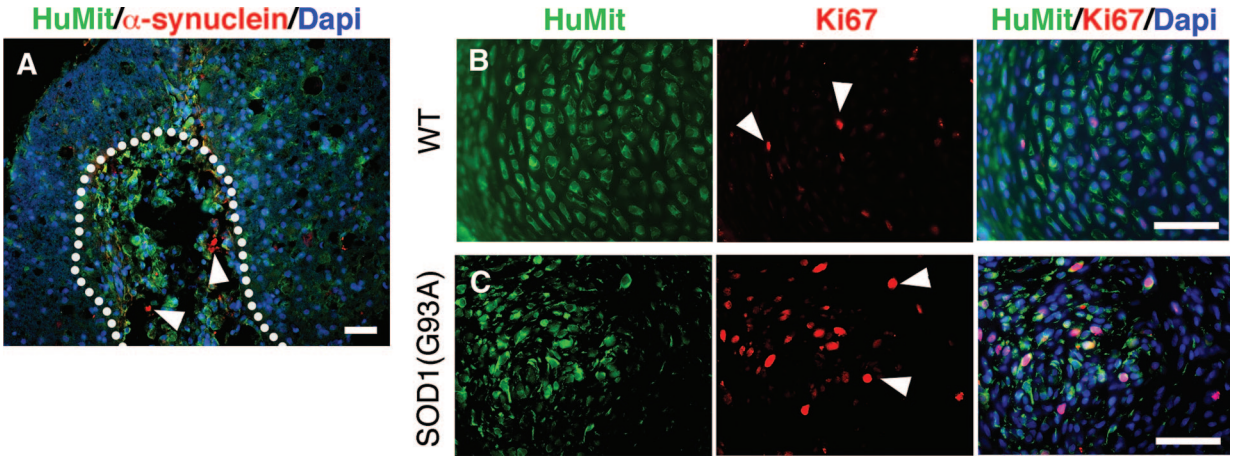

Figure 6. Lack of iPSC-NP degeneration. **(A):** Cell loss, evidenced in the central area of the graft in one SOD1(G93A) rat at day 30, was not due to progenitor degeneration. Cells were coimmunolabeled against HuMit (in green) and α-synuclein (in red). Nuclei were counterstained with Dapi. Sporadic α-synuclein-positive bodies (white arrowheads) were detected nearing the area of graft loss (dashed perimeter), but the engrafted progenitors showed no sign of inclusion body formation (HuMit-positive/α-synuclein-negative). **(B, C):** Random HuMit-positive cells (in green) with Ki67-positive nuclei (in red) detected in the spinal cord of WT rats ($n = 3$) and SOD1(G93A) rats ($n = 4$) at day 30 post-transplantation. Arrowheads show cells coexpressing HuMit and Ki67 as detected from the left panels of **(B)** and **(C)**. Nuclei were counterstained with Dapi. Scale bars = 100 μm. Abbreviations: Dapi, 4′,6-diamidino-2-phenylindole; HuMit, human mitochondria; WT, wild-type.

cellular replacement in personalized medicine, and animal studies have shown that iPSC-based technology could be used to reverse these diseases. For instance, functional dopamine neurons effectively reversed pathological features of Parkinson's disease in mice and rats [22–26], but the efficacy depended on the source of pluripotent cells and the reprogramming strategy. For motor neuron diseases, only a few studies monitored the behavior of human pluripotent cells or progenitors after transplantation into animals. For instance, it has been shown that human NSCs transplanted into the lumbar spinal cord of G93 mice [27] and SOD1 rats [28] can differentiate into neurons and delay ALS onset, probably via the release of growth factors necessary for the survival of host motoneurons. Likewise, Wyatt et al. successfully transplanted motoneuron progenitors derived from human embryonic stem cells into rodent models of motor neuron loss [29]. Another recent study showed that neural precursors isolated from adult human spinal cord proliferate in vitro and that upon transplantation into the injured rat spinal cord they give rise to neurons and glial cells [30]. The "trophic alternative" was also studied in mouse models of ALS transplanted with human glial-restricted progenitors [31]. These progenitors successfully engrafted in the cervical spinal cord of mice and differentiated into glial subtypes but did not form tumors. However, disease progression was not modified, proving once again the complexity of ALS pathology and the difficulty of conceiving a feasible cell therapy for this disease. In addition, it is difficult to obtain neural progenitors from the adult human central nervous system or from human embryos because of ever-present ethical concerns.

iPSC-based technology remains an attractive approach for ALS therapy of neurodegenerative diseases, such as Parkinson's disease. But important issues have to be resolved: source of the cells, age of donors, transplantation technology, optimal time of intervention, reprogramming protocols, immunosuppressive treatment, and risk of teratoma formation. Successful iPSC-based therapy must optimize these factors, and it will likely require the use of drugs to support iPSC survival and differentiation. Here, we report that human iPSC-NPs transplanted into a pro-ALS environment survive and give rise to mature neurons, which were detected 60 days after transplantation. The average age of disease onset in SOD1(G93A) rats was reported to be approximately 115 days [32, 33]. We observed initiation of motoneuron loss in SOD1 spinal cord around 95 days of age and symptoms of limb paralysis around 150 days of age. Our data show that neuronal differentiation was progressive and without signs of substantial cell death or morphological indicators of a degenerative process, at least at the time points we analyzed. iPSC-NPs progressively lost their endogenous expression of nestin and acquired a mature neuronal MAP2 phenotype by day 60 post-transplantation, passing through a preneuronal DCX phenotype identified on day 30 but not observed on day 60 (data not shown). The presence of MAP2-negative precursors together with MAP2-positive cells (Fig. 4B) and the coexistence of nestin-positive and nestin-negative clusters inside the graft on day 60 (data not shown) suggest that undifferentiated progenitors were still present in the graft. Similar observations were made in many iPSC transplantation experiments, including in parkinsonian rats, in which proliferating nestin-positive progenitors were found among mature functional dopaminergic neurons [23]. Analyses at later time points are needed to assess whether these cells continue to give rise to other neurons or whether they can persist and form teratoma-like structures. Although we detected rare and transient Ki67-positive cells in graft sections on day 30 in two animals, we did not observe tumors or Oct4-positive staining on day 60 (data not shown), and no transplanted rat died during the follow-up period from causes other than surgical stress. Interestingly, iPSC-NP morphology changed along the localization of transplanted cells in the gray matter. This morphologic modification was paralleled by fiber projection toward the white matter and the interior of the horn. Xenografting experiments on cellular repair in Parkinson and Huntington animal models have shown that the growth and specification of transplanted neurons are directed by the host brain [34], so similar mechanisms could explain our observations. Although we did not study the in vivo performance of engrafted cells and their further specification, we demonstrate that human iPSC-NPs can survive and give rise

to mature neurons, including some with motor neuron morphology. The difference we observed in the kinetics of iPSC-neural cell differentiation between WT and SOD1 rats may be explained by the inflammatory environment of the engrafted cells. As suggested by Trujillo et al. [35], the differentiation fate of human iPSCs into neurons and astrocytes could be influenced by the kallikrein-kinin system, which is involved in inflammation. We did not detect HuMit-positive cells expressing GFAP at any time in either WT or SOD1 rats, suggesting that the differentiation process was not directed toward astrocyte formation. This result is consistent with the absence of glial differentiation in vitro [17].

The yield of motor neurons generated from pluripotent stem cells varies from 0.5% to 50% [6]. In our paradigm, several key factors might have enhanced the differentiation into mature neurons/motoneurons in sufficient numbers. First, the progenitors were transplanted in a presymptomatic SOD1 model, in which human pluripotent cells survive better than in symptomatic models [36], probably because of a less toxic environment around the grafts during the presymptomatic stage. Moreover, we injected large amounts of reprogrammed cells directly in the lumbar spinal cord. Although this procedure is invasive, it can ensure better migration to the sites of motoneuron degeneration.

Finally, we point out that we did not evaluate parameters such as axonal outgrowth or synaptogenesis of the engrafted iPSC-derived neurons. This will be done in the future by applying an in vivo bioassay during longer observation periods in order to evaluate disease evolution and related changes.

Conclusion

We have demonstrated the feasibility of transplanting human iPSC-NPs into an ALS environment. Human iPSC-NPs successfully engraft, survive, and differentiate into mature neurons in the spinal cord of an ALS rat model. Our study provides an optimistic perspective for iPSC-based therapy of ALS and encourages further investigation.

Acknowledgments

We thank Robert de Decker for excellent technical help in animal experimentation, Michèle Authelet and Valérie Suain for assistance in histology experiments, Thomas Lemonnier for generation and in vitro characterization of iPSCs, Stéphane Blanchard for lentiviral vector production, and Jean-Michel Heard for help and advice. This work was funded by Latran Foundation, Institut Pasteur Paris, INSERM, Zone 62 du Lions Club District 112D Belgium, the Group François Ghigny (Belgium), and Marc Dausimont from Aplanos Running Team (Belgium).

Author Contributions

I.R.P.: conception and design, performance of experiments, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; C.N: collection and assembly of data, data analysis and interpretation, final approval of manuscript; S.L. and S.K.: spinal cord neurosurgery, final approval of manuscript; G.B.: iPSC culture, final approval of manuscript; V.D.: data analysis and image processing, final approval of manuscript; D.B. and R.P.: conception and design, financial support, provision of study material, data interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Mitchell JD, Borasio GD. Concise review: Amyotrophic lateral sclerosis. Lancet 2007; 369:2031–2041.

2 Cheah BC, Vucic S, Krishnan AV et al. Concise review: Riluzole, neuroprotection and amyotrophic lateral sclerosis. Curr Med Chem 2010;17:1942–1999.

3 Wijesekera LC, Leigh PN. Concise review: Amyotrophic lateral sclerosis. Orphanet J Rare Dis 2009;4:3.

4 Rosen DR, Siddique T, Patterson D et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 1993;362:59–62.

5 Traub R, Mitsumoto H, Rowland L. Concise review: Research advances in amyotrophic lateral sclerosis, 2009 to 2010. Curr Neurol Neurosci Rep 2011;11:67–77.

6 Lindvall O, Kokaia Z. Concise review: Stem cells in human neurodegenerative disorders: Time for clinical translation? J Clin Invest 2010; 120:29–40.

7 Thonhoff JR, Ojeda L, Wu P. Concise review: Stem cell-derived motor neurons: Applications and challenges in amyotrophic lateral sclerosis. Curr Stem Cell Res Ther 2009;4:178–199.

8 Mitrecić D, Nicaise C, Gajović S et al. Distribution, differentiation, and survival of intravenously administered neural stem cells in a rat model of amyotrophic lateral sclerosis. Cell Transpl 2010;19:537–548.

9 Durnaoglu S, Genc S, Genc K. Concise review: Specific pluripotent stem cells in neurological diseases. Stem Cells Int 2011;2011: 212487.

10 Dimos JT, Rodolfa KT, Niakan KK et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 2008;321: 1218–1221.

11 Karumbayaram S, Novitch BG, Patterson M et al. Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. Stem Cells 2009; 27:806–811.

12 Ebert AD, Yu J, Rose FF, Jr., et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. Nature 2009;457: 277–280.

13 Mitne-Neto M, Machado-Costa M, Marchetto MC et al. Downregulation of VAPB expression in motor neurons derived from induced pluripotent stem cells of ALS8 patients. Hum Mol Genet 2011;20:3642–3652.

14 Xu L, Tan YY, Ding JQ et al. Concise review: The iPs technique provides hope for Parkinson's disease treatment. Stem Cell Rev 2010;6:398–404.

15 Bilican B, Serio A, Barmada SJ et al. Mutant induced pluripotent stem cell lines recapitulate aspects of TDP-43 proteinopathies and reveal cell-specific vulnerability. Proc Natl Acad Sci USA 2012;109:5803–5808.

16 Egawa N, Kitaoka S, Tsukita K et al. Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci Transl Med 2012;4:145ra104.

17 Lemonnier T, Blanchard S, Toli D et al. Modeling neuronal defects associated with a lysosomal disorder using patient-derived induced pluripotent stem cells. Hum Mol Genet 2011;20:3653–3666.

18 Brion JP, Guilleminot J, Couchie D et al. Both adult and juvenile tau microtubule-associated proteins are axon specific in the developing and adult rat cerebellum. Neuroscience 1988;25:139–146.

19 Bellin M, Marchetto MC, Gage FH et al. Induced pluripotent stem cells: The new patient? Nat Rev Mol Cell Biol 2012;13:713–726.

20 Park IH, Arora N, Huo H et al. Disease-specific induced pluripotent stem cells. Cell 2008;134:877–886.

21 Baker M. Neurons from reprogrammed cells. Nat Methods 2011;8:905–909.

22 Rhee YH, Ko JY, Chang MY et al. Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a

rat model of Parkinson disease. J Clin Invest 2011;121:2326–2335.

23 Cai J, Yang M, Poremsky E et al. Dopaminergic neurons derived from human induced pluripotent stem cells survive and integrate into 6-OHDA-lesioned rats. Stem Cells Dev 2010;19:1017–1023.

24 Hargus G, Cooper O, Deleidi M et al. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proc Natl Acad Sci USA 2010;107: 15921–15926.

25 Kriks S, Shim JW, Piao J et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. Nature 2011;480:547–551.

26 Swistowski A, Peng J, Liu Q et al. Efficient generation of functional dopaminergic neurons from human induced pluripotent stem cells under defined conditions. Stem Cells 2010; 28:1893–1904.

27 Yan J, Xu L, Welsh AM et al. Combined immunosuppressive agents or CD4 antibodies prolong survival of human neural stem cell grafts and improve disease outcomes in amyotrophic lateral sclerosis transgenic mice. Stem Cells 2006;24:1976–1985.

28 Xu L, Yan J, Chen D et al. Human neural stem cell grafts ameliorate motor neuron disease in SOD-1 transgenic rats. Transplantation 2006;82:865–875.

29 Wyatt TJ, Rossi SL, Siegenthaler MM et al. Human motor neuron progenitor transplantation leads to endogenous neuronal sparing in 3 models of motor neuron loss. Stem Cells Int 2011;2011:207230.

30 Mothe AJ, Zahir T, Santaguida C et al. Neural stem/progenitor cells from the adult human spinal cord are multipotent and self-renewing and differentiate after transplantation. PLoS One 2011;6:e27079.

31 Lepore AC, O'Donnell J, Kim AS et al. Human glial-restricted progenitor transplantation into cervical spinal cord of the SOD1 mouse model of ALS. PLoS One 2011;6:e25968.

32 Howland DS, Liu J, She Y et al. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). Proc Natl Acad Sci USA 2002;99:1604–1609.

33 Nicaise C, Mitrecic D, Demetter P et al. Impaired blood–brain and blood–spinal cord barriers in mutant SOD1-linked ALS rat. Brain Res 2009;1301:152–162.

34 Isacson O, Deacon TW, Pakzaban P et al. Transplanted xenogeneic neural cells in neurodegenerative disease models exhibit remarkable axonal target specificity and distinct growth patterns of glial and axonal fibres. Nat Med 1995;1:1189–1194.

35 Trujillo CA, Negraes PD, Schwindt TT et al. Kinin-b2 receptor activity determines the differentiation fate of neural stem cells. J Biol Chem 2012;287:44046–44061.

36 Knippenberg S, Thau N, Schwabe K et al. Intraspinal injection of human umbilical cord blood-derived cells is neuroprotective in a transgenic mouse model of amyotrophic lateral sclerosis. Neurodegener Dis 2012;9: 107–120.