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Survival and Differentiation of Transplanted Neural Stem Cells Derived from Human Induced Pluripotent Stem Cells in a Rat Stroke Model

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Abstract

Introduction—Although administration of various stem cells has shown promise in stroke models, neural stem cells (NSCs) derived from human induced pluripotent stem cells (iPSCs) have advantages over other cell types. We asked if these cells could survive, differentiate, and improve stroke recovery in an ischemic stroke model.

Methods—Human iPSCs were induced in vitro to an early NSC stage. One week after focal cerebral ischemia, 20 rats received cells or vehicle by intracerebral injection. Graft cell fate, infarct volume, and behavioral deficits were assessed.

Results—Graft cells were found in 8 of the transplanted rats (80%), with estimated mean graft cell numbers nearly double the amount transplanted one month later. Graft cells also expressed markers of NSCs in 5 rats (63%), neurons in all 8 rats (100%), rare astrocytes in 4 rats (50%), and signs of proliferation in 4 rats (50%), but no tumor formation was observed. Stroke volume and behavioral recovery were similar between groups.

Discussion—To our knowledge this is the first report of transplantation of NSCs derived from human iPSCs in a stroke model. Human iPSC-derived NSCs survived in the post-ischemic rat brain and appeared to differentiate, primarily into neurons. This cell transplantation approach for stroke appears to be feasible, but further optimization is needed.

Introduction

Stroke is a major cause of disability, with few effective treatments available to improve recovery.[1] Cell transplantation is a promising potential treatment to improve stroke recovery, and multiple cell types have shown promise in stroke models.[2] Neural stem cells (NSCs) are the precursors of neurons and glia, the primary cells lost with stroke. NSCs may be obtained from embryonic or fetal tissue, but ethical concerns and the potential need for long-term immunosuppression to prevent graft rejection are problems that may limit their utility. NSCs may also be derived from induced pluripotent stem cells (iPSCs), which behave in a similar manner to embryonic stem cells in vitro, but are created from adult cells such as skin fibroblasts. These cells could be obtained from the stroke patient, avoiding both

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ethical concerns and the need for immunosuppression.[3] We asked if NSCs derived from human iPSCs could survive, differentiate, and improve stroke recovery after transplantation into a rat model of ischemic stroke.

Methods

The human iPS-DF6-9-9T cell line was chosen because it was created from postnatal skin fibroblasts and free of transgenes,[4] and they were differentiated to an early NSC phenotype as described,[5] with the following modifications. The cells were expanded on a feeder layer of irradiated mouse embryonic fibroblasts with a daily change of embryonic stem cell medium (ESM) that consisted of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), 20% serum replacement (Gibco), 1% Minimum Essential Medium Eagle: Non-Essential Amino Acids (MEM-NEAA, Gibco), 0.1 mM beta-mercaptoethanol (Sigma), 1 mM L-glutamine (Gibco), and 4 ng/ml fibroblast growth factor 2 (FGF2, R&D). Cells were passaged to new plates with 0.1–0.2 mg/ml dispase (Gibco) at 37°C for 1 min, and mechanically dissociated every 7 days.

The cells were directed in vitro for 21 days to the NSC phenotype of neuroepithelial cells expressing Pax6 and Sox1 but not Oct4. Cell colonies were suspended in ESM without FGF2, and cultured for 4 days in 25 cm2 tissue culture flasks (Nunclon) with a daily medium change. The cell colonies grew as floating clusters, while any remaining feeder cells adhered to the flask and were removed by transferring the clusters into new flasks. On day 4, the daily medium change was switched to neural induction medium (NIM) of DMEM/F12, 1% MEM-NEAA, 1% N2 supplement (Gibco), and 2 ug/ml of heparin (Sigma). On day 7, the clusters were attached to plastic plates with the addition of 10% fetal bovine serum (Gibco) for 12 hours, and some clusters were attached to laminin (Sigma)-coated glass coverslips for immunostaining. Adherent colonies had NIM changed every other day and rosettes formed. On day 14, rosettes were mechanically separated from the surrounding flat cells and again grown as floating clusters in a flask with a change of NIM with 2% B27 supplement (Gibco) every other day. On day 19, the size of the clusters was reduced with accutase and trituration. On day 21, the cluster solution was well-mixed and a small portion immediately removed for counting. The clusters in that portion were dissociated to a single-cell suspension with accutase, and the concentration of live and dead cells was determined using trypan blue and a hemocytometer. The clusters were then suspended at a concentration of approximately 50,000 cells/ul in transplantation medium of NIM, 2% B27, and growth factors (brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and insulin-like growth factor 1, each at 1:10,000, and cyclic adenosine monophosphate at 1:1,000). Morphological analyses and immunostaining with markers for progenitors and more mature neural cells were performed during the course of in vitro differentiation to monitor consistency of differentiation.

All animal procedures were approved by the institutional animal care and use committee. For the procedures the rats were anesthetized with 1–2% inhaled isoflurane in oxygen, local bupivicaine 3 mg/kg and systemic buprenorphine 0.02 mg/kg were given subcutaneously (SC), and body temperature was maintained at 37°C with a rectal probe and heating pad feedback system. Ischemic stroke was induced in adult male Wistar rats (Charles River) weighing 275–325 grams by 30 minutes of intraluminal filament middle cerebral artery occlusion (MCAO) as previously described,[6] with modifications. Through a ventral neck incision, the left carotid bifurcation was exposed and the proximal end of the exposed portion of the common carotid artery (CCA) was permanently ligated. The distal CCA was temporarily ligated and an arteriotomy was made between the ligatures for insertion of a synthetic filament with the tip coated with silicone (Doccol). The filament was advanced up the internal carotid artery until resistance was felt at 15–20 mm distal to the carotid

bifurcation indicating MCAO. Anesthesia was maintained during the 30 minutes of MCAO, at which point the filament was removed, the distal CCA permanently ligated, the incision closed, and the rat kept warm during recovery.

Seven days following MCAO, rats were randomly assigned to treatment groups, placed in a stereotactic frame, and a small craniotomy was made at 0 mm anterior and 3 mm left of bregma. Approximately 250,000 cells in 5 ul of vehicle were loaded in a syringe with a 26 gauge needle, which was slowly inserted to a depth of 6 mm from the skull and left in place for 3 mins prior to the first injection. Boluses of 1 ul each were injected at five depths (6, 5, 4, 3, and 2 mm) relative to the skull at a rate of 1 ul/min, and the needle was left in place 3 mins after the last injection prior to removal. The incision was closed, and the rat kept warm during recovery. The control rats had identical injections of vehicle without cells. Cyclosporine 10 mg/kg/day SC was given to all the rats (cell-treated and control) for 7 days starting 2 days prior to transplantation, followed by 100 mg/ml in drinking water through the survival period.

Behavioral testing was performed, blind to group assignment, twice at each of 4 time points relative to MCAO: prior (baseline), 1, 3, and 5 weeks. The elevated body swing test assessed motor symmetry by gently lifting the rat by the tail and recording the direction of lateral body flexion through 20 trials.[7] The cylinder test assessed symmetry of independent forelimb use as the rat explored the walls of a transparent plastic cylinder for 50–100 total touches.[8] The adhesive removal test assessed symmetry of forelimb sensation when small adhesive dots 9.5 mm in diameter were simultaneously placed on both dorsal forepaws, and the difference in removal time was recorded up to a 10 min trial.[9]

Five weeks after MCAO, rats were anesthetized with isoflurane in oxygen and perfused with saline followed by 4% paraformaldehyde (PFA). The brain was removed and stored in 4% PFA at 4°C for 1 day, followed by phosphate-buffered sa line (PBS) with 30% sucrose. After freezing the brain with dry ice, 30 um coronal sections were made with a sliding microtome through the striatum starting at the anterior corpus callosum. Sections were stored in PBS with 30% sucrose and 30% ethylene glycol at 4°C. Ten serial sections 360 um apart were stained with cresyl violet and measured with ImageJ. The noninfarcted ipsilesional hemisphere volume was divided by the area of the contralesional hemisphere for the stroke volume, expressed as a percentage relative to the contralesional hemisphere. Immunohistochemistry was performed to identify graft cells and their differentiation status using antibodies to human cells (HuNu, Chemicon, 1:300), and double-labeling for markers of neural stem cells (Nestin, Chemicon, 1:500), markers of neurons (Microtubule-associated protein 2, MAP2, Chemicon, 1:1,000 and Neuron-specific class III beta-tubulin, TUJ1, Fisher, 1:1,000), a marker of astrocytes (Glial fibrillary acidic protein, GFAP, DAKO, 1:5,000), a marker of oligodendrocytes (Myelin basic protein, MBP, Chemicon, 1:200), and a marker of cell proliferation (Ki67, Fisher, 1:200). Fluorescent secondary antibodies (Cy3 and AF488, Invitrogen, 1:1,000) and Hoechst (Invitrogen, 1:400) were applied, and double-labeling was confirmed with a Nikon 80i Upright Confocal Microscope.

The number of labeled cells was stereologically estimated using the optical fractionator procedure (Stereo Investigator, MicroBrightField). Visible grafts were outlined on every 12th serial section and systematic sampling was performed of labeled cells, with a counting frame of 30 × 30 um, dissector height of 10 um, guard zones of 2 um, an average of 10 sampling sites per section, and 200–300 cells counted per rat. Double labeling was confirmed with a Nikon 80i Upright Confocal Microscope, and quantified by counting 100 consecutive cells per marker for each rat using the cell counter function of ImageJ on a single-plane 40X image centered on the center of the graft.

Optical densitometry of fluorescent markers was determined on 4X images of the graft and the matching contralateral brain area that were captured under identical light conditions with a Nikon E600 fluorescence microscope, Photometrics CoolSnap HQ2 camera, and NIS-Elements software. The auto threshold function of ImageJ was used to mark pixels over a minimum intensity on each graft image, the same setting were then applied to the paired contralateral image, and the integrated density (ID) was measured for the marked pixels as the product of the area and the mean intensity. Graft versus contralateral ID values were then compared to correct for variability in background staining.

Data are presented as mean ± s.e.m., stroke volumes were compared using the Mann–Whitney test, and behavioral scores were compared with one-way repeated measures ANOVA.

Results

One month after transplantation we found HuNu+ graft cells in 8 of 10 (80%) rats, including in brain tissue immediately adjacent to the ragged edge of infarct cavities (Fig 1 and 2). The mean unbiased stereological estimate of surviving graft cells was 477,967 (±172,736), which was nearly double the number transplanted. Graft cells expressed the neuronal markers βIII-tubulin and MAP2 in all 8 rats (100%), which was seen in 41 (±10)% of graft cells, some of which also extended neurite-like processes (Fig 1). The astrocyte marker GFAP was expressed in 5 (±5)% of graft cells in 4 rats (50%) (Fig 2). No graft cells expressed the oligodendrocyte marker MBP. No tumors were seen, but 32 (±12)% of graft cells also expressed the NSC marker Nestin in 5 rats (63%) (Fig 2).

In 4 rats (50%) grafts demonstrated hypercellularity (Hoechst graft versus contralateral ID, $p=0.004$) and rosette formation, similar in appearance to those formed in vitro, as well as expression of the proliferation marker Ki67, which was seen in 13 (±5)% of graft cells, primarily in the central portion of the rosettes (Fig 2). Significant migration of graft cells was not seen. No seizures or other adverse effects were seen in the rats during the month following transplantation. The cell and vehicle treated groups did not exhibit significant differences in stroke volume or behavioral recovery ($p>0.05$ for both, Fig 3).

Discussion

We found that transplanted NSCs derived from human iPSCs survive in the post-ischemic rat brain, including in tissue adjacent to cavitated infarction, and appear to differentiate primarily into neurons. The cells did not reduce stroke volume or improve behavioral recovery during the month following transplantation.

We injected cells directly into the brain to maximize delivery to the peri-infarct tissue, but the optimal route of transplantation of the various cell types is currently unclear.[10] The lack of functional improvement may be attributed to multiple factors, including the optimal timing of transplantation following stroke, the optimal dose of cells to use, and many variables of the cells themselves such as their source, culture protocol, and differentiation stage.[10] In addition, human pluripotent stem cell-derived neural progenitors may require months for maturation before they could contribute to functional improvement.[11] It is possible that our cells require more time to mature and integrate into neuronal networks than one month before they correct functional deficits, which will be our future endeavor.

We recently performed a systematic review of human NSC transplantation in animal stroke models.[12] Similar to the preset study, we found the most frequent time point for transplantation was one week after stroke, and that the most common route of cell delivery was intracerebral injection; unlike our study the most common origin of the cells used was

fetal brain tissue. Most of the studies found transplanted cells in the brain with histology, and the grafted cells expressed markers of neurons more often than other cell types, as we also found. Like our results most studies did not a find a reduction in stroke volume. Unlike our findings all studies that reported behavioral testing found behavioral rescue with grafting on at least one test. We concluded that the available data support the promise and need for further development of NSC transplantation as a future treatment for stroke patients, but also suggest the possibility of publication bias.

Tumor formation has been reported with transplantation of undifferentiated murine, but not human, iPSCs in rodent stroke models.[13, 14] We did not see tumor formation, but evidence of continued proliferation of graft cells one month after transplantation suggests that the differentiation stage we used of this particular cell line might be too immature to be safe. There may be a trade-off, however, in that these immature cells showed robust survival in the hostile environment of post-ischemic brain tissue, even in close proximity to the ischemic border zone, which may not be the case with more mature neural cells. This could be particularly advantageous in light of the fact that we did not observe significant migration of the graft cells, so they may need to be placed close to the area of infarction.

To our knowledge, this is the first report of transplantation of NSCs derived from human iPSCs in a stroke model. This cell transplantation approach for stroke appears feasible, but the variables of the regimen require optimization to allow for a reliable assessment of the potential to improve recovery. Further studies are needed to show benefit with this cell type by testing key variables starting with the NSC differentiation stage, the location and orientation of intracerebral graft placement, and the timing of transplantation after stroke.

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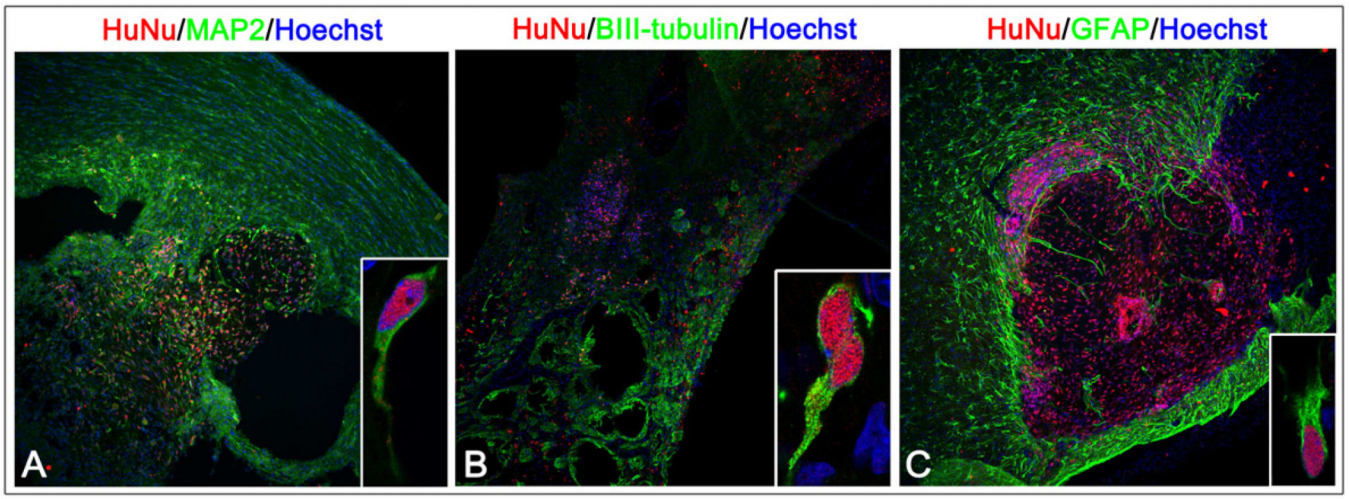

Fig. 1.

10X and 100X magnification views of HuNu+ graft cells (red, A/B/C) also expressing the neuronal markers MAP2 (green, A) and βIII-tubulin (green, B), or the astrocyte marker GFAP (green, C) in the post-ischemic rat brain one month after transplantation.

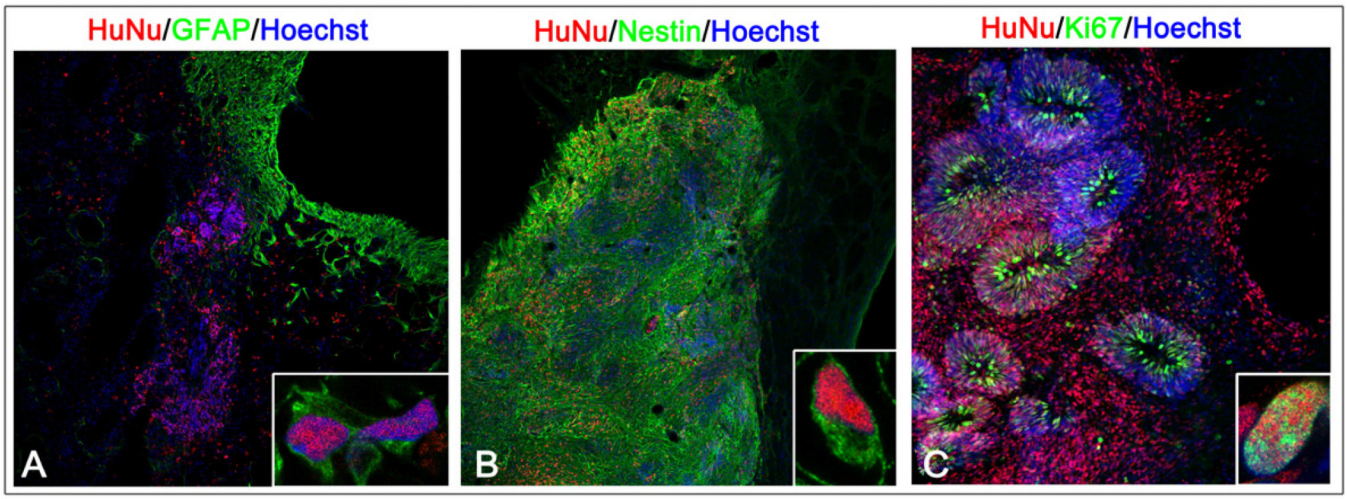

Fig. 2.

10X and 100X magnification views of HuNu+ graft cells (red, A/B/C) also expressing the astrocyte marker GFAP (green, A), the NSC marker Nestin (green, B), or the cell division marker Ki67 (green, C).

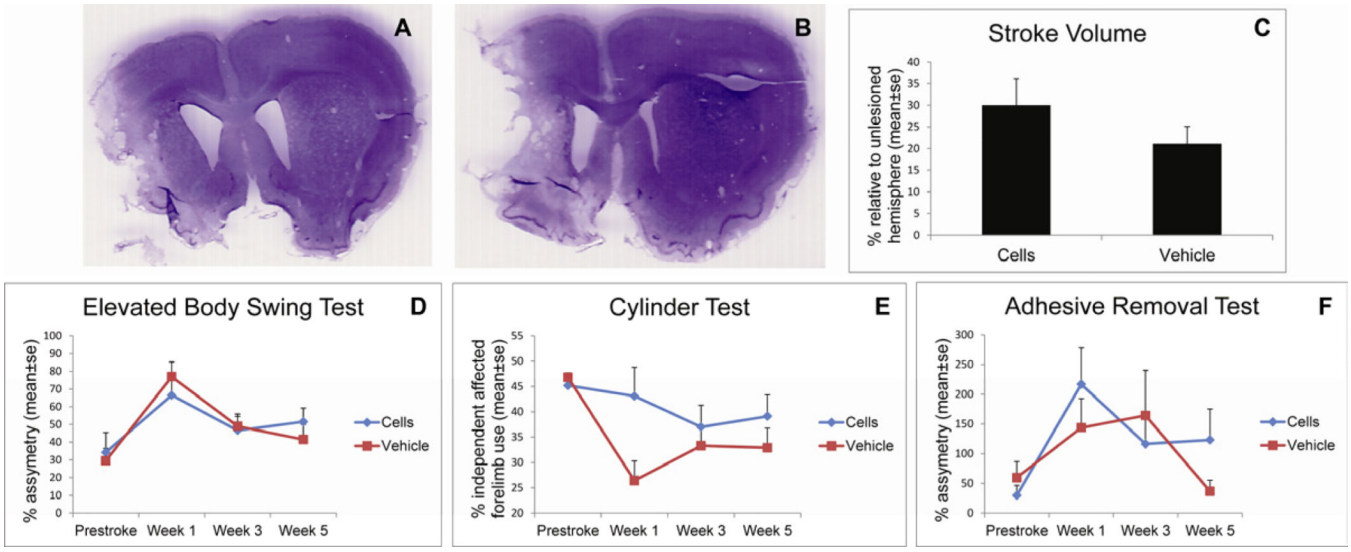

Fig. 3.

Representative cerebral infarcts of cell (A) and vehicle treated (B) rats, and the group mean stroke volumes expressed as a percentage of the unlesioned hemisphere (C). Behavioral testing results (D–F), which did not demonstrate a significant difference in recovery between groups.