Published in final edited form as:

J Comp Neurol. 2014 August 15; 522(12): 2707–2728. doi:10.1002/cne.23578.

Human neural progenitor cells generated from induced pluripotent stem cells can survive, migrate, and integrate in the rodent spinal cord

Dhruv Sareen^{1,2,*}, Geneviève Gowing¹, Anais Sahabian¹, Kevin Staggenborg¹, Renée Paradis¹, Pablo Avalos¹, Jessica Latter¹, Loren Ornelas¹, Leslie Garcia¹, and Clive N. Svendsen^{1,2,*}

¹Regenerative Medicine Institute, Cedars-Sinai Medical Center, AHSP 8th floor, 8700 Beverly Blvd., Los Angeles, CA 90048, USA

²Department of Biomedical Sciences, Cedars-Sinai Medical Center, AHSP 8th floor, 8700 Beverly Blvd., Los Angeles, CA 90048, USA

Abstract

Transplantation of human neural progenitor cells (NPCs) into the brain or spinal cord to replace lost cells, modulate the injury environment or create a permissive milieu to protect and regenerate host neurons is a promising therapeutic strategy for neurological diseases. Deriving NPCs from human fetal tissue is feasible, though problematic issues include limited sources and ethical concerns. Here we describe a new and abundant source of NPCs derived from human induced pluripotent stem cells (iPSCs). A novel chopping technique was used to transform adherent iPSCs into free-floating spheres that were easy to maintain and were expandable (EZ spheres) (Ebert et al., 2013). These EZ spheres could be differentiated towards NPC spheres with a spinal cord phenotype using a combination of all-trans retinoic acid (ATRA) and epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) mitogens. Suspension cultures of NPCs derived from human iPSCs or fetal tissue have similar characteristics, though they were not similar when grown as adherent cells. In addition, iPSC-derived NPCs (iNPCs) survived grafting into the spinal cord of athymic nude rats with no signs of overgrowth and with a very similar profile to human fetal-derived NPCs (fNPCs). These results suggest that human iNPCs behave like fNPCs and could thus be a valuable alternative for cellular regenerative therapies of neurological diseases.

*Address correspondence to: Dhruv Sareen, PhD, Regenerative Medicine Institute and Department of Biomedical Sciences, Cedars-Sinai Medical Center, AHSP 8418, 8700 Beverly Blvd., Los Angeles, CA 90048, USA. Tel: (310) 248-8556; Fax: (310) 248-8555; dhruv.sareen@cshs.org, Clive N. Svendsen, PhD, Regenerative Medicine Institute and Department of Biomedical Sciences, Cedars-Sinai Medical Center, AHSP A8404, 8700 Beverly Blvd., Los Angeles, CA 90048, USA. Tel: (310) 248-8072; Fax: (310) 248-8066; clive.svendsen@cshs.org.

COMPETING INTERESTS: The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS: Conceived and designed the experiments: DS, GG, CNS. Performed the experiments: DS, GG, AS, KS, RP, PA, JL, LO, LG. Analyzed the data: DS, GG, CNS. Contributed reagents/materials/analysis tools: DS, GG, CNS. Wrote the paper: DS, GG, CNS.

INTRODUCTION

Fetal neural progenitor cells (fNPCs) can be isolated from different regions of the developing human brain, expanded in culture and then differentiated into neurons and glia (Conti and Cattaneo, 2010; Kriegstein and Alvarez-Buylla, 2009). We have previously shown that fNPCs transplanted into the brain, spinal cord or retina in animal models of disease can survive and migrate, and provide beneficial effects in some cases (Andres et al., 2011; Nichols et al., 2013; Wang et al., 2008). Moreover, we have genetically engineered these cells to produce therapeutic molecules for neuroprotection following transplantation in animal models of Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Ebert et al., 2008; Suzuki et al., 2007). Other groups have also generated and shown the potential of human fNPCs, which in some cases have been taken forward into Federal Drug Administration-approved clinical trials for a number of neurological disorders with no reported serious adverse effects (Azienda Ospedaliera Santa Maria et al., 2012; Glass et al., 2012; Neuralstem Inc. and Emory University, 2011; ReNeuron Limited, 2010; Robberecht and Philips, 2013; StemCells, 2006; StemCells, 2011; StemCells, 2012; Tamaki et al., 2002; Taupin, 2006). However, due to the limited supply, concerns of chromosomal aberrations (aneuploidies) during expansion (Sareen et al., 2009), and ethical concerns associated with the use of aborted human fetal tissues there is a pressing need for alternative sources.

Human pluripotent stem cells (hPSCs) including, embryonic stem cells (ESCs) derived from the blastocyst of a developing embryo and induced PSCs (iPSCs) derived from reprogrammed adult somatic cells have great potential for generating cells for use in regenerative and cell replacement strategies (Okano et al., 2013; Robinton and Daley, 2012; Yamanaka, 2012). They are essentially immortal allowing limitless cellular expansion and banking, and extremely plastic allowing differentiation into any cell type. Human iPSCs also offer an unprecedented opportunity for autologous transplantation, possibly circumventing the complexities surrounding immunological rejection with allogeneic human cell transplantation (Araki et al., 2013; Kaneko and Yamanaka, 2013; Liu et al., 2013; Okita et al., 2011c; Zhao et al., 2011). Human iPSCs can efficiently develop into neural cells (Chetty et al., 2013; Ebert et al., 2013; Kobayashi et al., 2012; Lee et al., 2012; Zhou et al., 2010), however, before iPSC-derived neural cells can be used in clinical transplantation trials they must 1) be shown to be safe, 2) maintain a normal cytogenetic status, 3) be devoid of residual pluripotent cells to avoid possible malignant tumor formation, 4) be reproducibly expanded in large numbers, and finally 5) survive and integrate into relevant central nervous system regions.

Neuronal replacement is one strategy to use for future clinical transplantation trials. However, in fact, astroglial cells are the most abundant cell type in the human brain and spinal cord and are now understood to be as important as neurons for brain function (Oberheim et al., 2006). They have also been implicated in a number of neurodegenerative diseases, with perhaps the best example being ALS, where glial dysfunction has been shown to lead to non-cell autonomous death of the motor neurons (Di Giorgio et al., 2007; Haidet-Phillips et al., 2011; Nagai et al., 2007; Yamanaka et al., 2008). Replacement of astrocytes (Lepore et al., 2011; Lepore et al., 2008; Nichols et al., 2013), either naive or secreting growth factors (Suzuki et al., 2007), has been shown to be beneficial in ALS models. We

have previously shown that fNPCs can give rise to astroglial progenitors that then differentiate to immature and mature astrocytes within the rodent brain and spinal cord over long time periods (Gowing et al., 2013; Klein et al., 2005; Suzuki et al., 2007; Svendsen et al., 1997). Human PSCs can also be directed into more mature astrocytes (Juopperi et al., 2012; Krencik et al., 2011; Yuan et al., 2013). While such PSC-derived mature astrocytes may survive transplantation, immature NPCs generated from iPSCs may provide cells that are easier to culture and expand *in vitro* and better suited to migrate, integrate and restore function *in vivo*.

Here, we report a new protocol for the production of expandable human iPSC-derived neural progenitor cells (iNPCs). These human iNPCs could be easily propagated over long-term as suspension cultures and had similar profile to human fNPCs. Following direct parenchymal injection, iNPCs successfully engrafted into athymic nude rats with no signs of tumor formation or overgrowth, and again appeared similar to fNPC transplants. Our results describe a new source of human neural progenitor cells that do not have the supply, expansion and ethical concerns of fNPCs, and hence could be ideal for stem cell-based therapeutic approaches.

METHODS

Generation of non-integrating human iPSCs using episomal plasmids

Apparently healthy human fibroblast cell lines (GM05400, 03814 and 02183) were obtained from the Coriell Institute for Medical Research, under their consent and privacy guidelines. All protocols were performed in accordance with the institutional review board's guidelines at the Cedars-Sinai Medical Center under the auspice IRB-SCRO Protocols Pro00028429 (Transplantation iPS derived human neural progenitors), Pro00021505, and Pro00032834. Upon iPSC generation at Cedars Sinai, they were renamed 00iCTR-n2, 15iCTR-n5, and 83iCTR-n1 to reflect catalog number, control line and clone number (Luong et al., 2011; Sareen et al., 2012). Fibroblasts were reprogrammed into virus-free iPSC lines using the Amaxa Human Dermal Fibroblast Nucleofector Kit to express episomal plasmids with 6 factors: *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and *p53* shRNA (Addgene) (Okita et al., 2011a). This method has a significant advantage over viral transduction, because exogenously introduced genes do not integrate and are instead expressed episomally in a transient fashion. Briefly, fibroblasts (0.8×10^6 cells per nucleofection) were harvested, centrifuged at 200g for 5 minutes, re-suspended carefully in Nucleofector® Solution (VPD-1001, Lonza) and the U-023 program was applied. All cultures were maintained under norm-oxygen conditions (5% O₂) during reprogramming, which further enhance the efficiency of iPSC generation. The media was kept on for 48 hours and gradually changed to chemically-defined mTeSR®1 medium containing small molecules to enhance reprogramming efficiency. The small molecules used were 1) sodium butyrate (0.5 mM), 2) glycogen synthase kinase 3β inhibitor of the Wnt/β-catenin signaling pathway (CHIR99021, 3 μM), 3) MEK pathway inhibitor (PD 0325901, 0.5 μM), 4) Selective inhibitor of TGF-β type I receptor ALK5 kinase, type I activin/nodal receptor ALK4 and type I nodal receptor ALK7 (A 83-01, 0.5 μM). Colonies with ES/iPSC-like morphology appeared 25–31 days later. Subsequently, colonies with the best morphology were transferred onto a feeder-

independent 6 BD Matrigel™ Matrix and maintained in mTeSR®1 medium. The iPSC clones were further expanded and cryopreserved according to previously published protocols (Yu et al., 2007).

Human iPSC characterization

Human iPSCs were rigorously characterized at the Cedars-Sinai iPSC core using several assays. G-Band karyotyping (see below) ensured normal a karyotype, and genomic DNA PCR confirmed the absence of episomal plasmid genes, as previously described (Muller et al., 2011; Okita et al., 2011b; Sareen et al., 2012). Pluripotency was assessed by immunostaining with surface and nuclear pluripotency markers for subsequent flow cytometry quantification (> 80% SSEA4 and Oct3/4 double positivity), by quantitative RT-PCR of endogenous pluripotency genes, and by gene-chip and bioinformatics-based PluriTest assays. Spontaneous embryoid body differentiation confirmed the capacity to form all germ layers.

Karyotype

Spheres were incubated in Colcemid (100 ng/mL; Life Technologies) for 30 minutes at 37°C and then dissociated using TrypLE for 10 minutes. They were then washed in phosphate buffered saline (PBS) and incubated at 37°C in 5mL hypotonic solution (1g KCl, 1g Na Citrate in 400mL water) for 30 minutes. The cells were centrifuged for 2.5 minutes at 1500 RPM and resuspended in fixative (methanol: acetic acid, 3:1) at room temperature for 5 minutes. This was repeated twice, and finally cells were resuspended in 500 µl of fixative solution and submitted to the Cedars-Sinai Clinical Cytogenetics Core for G-Band karyotyping.

EZ sphere generation for astrocyte formation

A 6-well plate of iPSCs was cultured to ~ 80% confluence and differentiating colonies were removed prior to generating neural stem cell aggregates (termed EZ spheres). To generate EZ spheres, media was aspirated, iPSC colonies were washed with PBS, treated with dispase (1 mg/mL) at 37°C for 15 minutes, gently washed with PBS, and gently collected in Stemline Neural Expansion media (Sigma) with a 5 ml pipette into a 15mL conical tube. After the primarily intact colonies settled to the bottom by gravity, media was aspirated and the colonies were resuspended in Stemline media supplemented with epidermal growth factor (EGF; 100 ng/ml; Millipore), fibroblast growth factor-2 (FGF-2; 100 ng/ml; Millipore), and heparin (5 µg/ml; Sigma) (termed StemHi E/F/H) and placed into one T75 flask coated with poly-HEMA to prevent attachment at 20% O₂ in 37°C incubator. Fresh media was replaced after the first 2 days of EZ sphere generation and subsequently every 3–4 days. The spheres were maintained in suspension, passaged by chopping with an automated tissue chopper into 200 µm chunks every 7–10 days as described previously (Ebert et al., 2013; Svendsen et al., 1998), and were efficiently cryopreserved and thawed for banking. EZ spheres after passage 10 are a stable population of neural stem cell aggregates with an excellent propensity for directed differentiation towards an astrocyte lineage (see below).

Neural progenitor cell generation from iPSC-derived EZ spheres

EZ spheres generated from iPSCs can be differentiated to a culture of neural progenitor cells in suspension (iNPCs^{SU}), with astroglial predisposition. After EGF/FGF2/heparin withdrawal, EZ spheres were caudalized using retinoic acid RA (0.5μM) in Neural Induction Media (NIM) (DMEM/F12, 1% NEAA, 1% N2, heparin 2μg/ml; Sigma). This media was replaced every 2 days for the next 11 days, after which a stable population of iNPCs^{SU} was reintroduced into StemHi E/F/H for expansion by weekly chopping (similar to EZ spheres). The iNPCs^{SU} maintain their proliferative potential and astroglial generation propensity for 26–30 passages and can be efficiently cryopreserved. In addition, an adherent format of iNPCs grown as adherent cultures and termed iNPCs^{AD}, were generated by accutase dissociation of EZ spheres for plating on growth-factor reduced Matrigel (Corning) at a density of 10,000 cells/cm² in StemHi E/F/H and passaged weekly using TrypLE (Life Technologies). For differentiation to astrocytes, iNPCs^{SU} were dissociated to single cells with accutase (BD Biosciences) or iNPCs^{AD} harvested with TrypLE were plated on poly-L-ornithine/Matrigel coated glass coverslips at 25000 cells/cm² in NIM for 7–21 days.

Fetal-derived human neural progenitors

Human fetal neural progenitor cells (fNPCs) isolated from 8 week old fetal cortex were maintained and expanded as free floating spheres as previously described (Sareen et al., 2009; Svendsen et al., 1997; Svendsen et al., 1998). Fetal NPC spheres were maintained in Stemline media supplemented with EGF (100 ng/ml), leukemia inhibitory factor (LIF; 100 ng/ml), Anti-Anti penicillin-streptomycin (1%, Life Technologies) and passaged by mechanical chopping (Svendsen et al., 1998). Following expansion, cells were prepared for transplantation at p28.

Cryopreservation

Spheres were collected and settled by gravity in a 15mL conical tube. The media was aspirated and the spheres were resuspended in serum-free, 8.7% DMSO-supplemented cell freezing media (Sigma). Cells were frozen at –80°C in an isopropyl alcohol chamber for 24 hours, followed by long-term storage in liquid nitrogen.

Antibody Characterization

Antibodies utilized in this study for immunocytochemical and histological analyses are summarized in Table 1 and have been tested for specificity. The *anti-A2B5 monoclonal* antibody (MAB312; Clone A2B5-105; Millipore) has been characterized by others to recognize A2B5 positive cells in fluorescence activated cell sorting (Windrem et al., 2004) and immunocytochemistry (Jiang et al., 2013). The *anti-Aldh1L1 monoclonal* antibody (73–140; clone N103/39; Antibodies, Inc.) recognizes a specific band at a molecular weight of 100 kDa on adult rat brain immunoblots (manufacturer's technical information). The *anti-Aquaporin 4 (AQP4)* polyclonal antibody (HPA014784, Sigma) has been validated by the manufacturer with two or more antibodies showing similar staining patterns consistent with gene characterization data and a band of predictive size ~37 KDa observed on immunoblot obtained from various cell lysates. The *GLAST (EAAT-1)* polyclonal antibody has been characterized by the manufacturer with predictive bands obtained at 60 and 120 kDa on

immunoblots. The *anti-Nestin* (AB5922; Millipore) polyclonal antibody corresponds to a detected 200–220-kDa band in human and monkey brain cell lysates (Messam et al., 2000). The *anti-GFAP* (Z0334; Dako) polyclonal antibody has been solid-phase absorbed with human and cow serum proteins and shows one distinct precipitate (GFAP) with cow brain extract via Coomassie brilliant blue (manufacturer's technical information) and labels a major band at the expected 50kDa on Western blots of mouse retina (Smith et al., 1997). The *STEM123* (Stem Cells, Inc.) monoclonal antibody reacts specifically with human GFAP but not rodent cells by immunohistochemistry (Gowing et al., 2013). Moreover, laboratory testing has shown that co-localization of signal is obtained by immunocytochemistry when using *STEM123* and *anti-GFAP* antibodies (Z0334; Dako) on human cells. The *anti-Ki67* (ab27619; clone SP6, Abcam) monoclonal antibody is a FITC conjugated equivalent to ab16667. Ab16667 recognizes a single band at 359 kDa on immunoblots from cell lysates (Manufacturer's technical information) and an increase in Ki67 labeling has been previously shown to correlate with increased BrdU labeling, another marker of cell proliferation (Bonzo et al., 2012). The *anti- β III-tubulin* (Tuj1, Clone SDL.3D10, T8660, Sigma) monoclonal antibody was shown to specifically recognize the protein on immunoblots (Lee et al., 1990). The *anti-S100B* (S2532; Clone SH-B1, Sigma) monoclonal antibody has been extensively characterized by immunohistochemistry and western blotting (20 kDa band in mouse homogenates) (Liao et al., 2008). The *STEM101* (Stem Cells, Inc.) monoclonal antibody reacts specifically with the Ku80 Protein located in nuclei of human cells only. It has been shown to specifically label the nuclei of human cells, but not rodent or monkey cells by immunohistochemistry (Mattis et al., 2014; Salazar et al., 2010). The *STEM121* (Stem Cells, Inc.) has been shown to specifically recognize the cytoplasm of human but not rodent cells or primate cells by immunohistochemistry (Cummings et al., 2005; Guzman et al., 2007; Kelly et al., 2004; Kriks et al., 2011; Mattis et al., 2014; Piltti et al., 2013; Salazar et al., 2010; Uchida et al., 2012). All pluripotency antibodies (OCT3/4; SOX2, NANOG, SSEA4, TRA-1-60, TRA-1-81; Table 1) have been extensively tested and validated in studies for immunocytochemistry (Sareen et al., 2013). The OCT3/4-PE conjugated antibody (561556; BD Biosciences) and SSEA4-AF647 conjugated antibody (560796; BD Biosciences) have been extensively tested for flow cytometry applications (Sareen et al., 2013).

Immunocytochemistry

The spheres were dissociated using accutase, and plated on coverslips at a density of 70,000 cells/well in Stemline media with appropriate mitogens for differentiation. Following differentiation in culture, plated cells were fixed with paraformaldehyde (PFA), rinsed in PBS, blocked in 5–10% goat or donkey serum with 0.2% Triton X-100 and incubated with primary antibodies; OCT3/4, NANOG, SOX2, SSEA4, TRA-1-60, TRA-1-81, GFAP, Ald1hL1, S100B, A2B5, Nestin, and Tuj1 at dilutions indicated in Table 1. After 1 hour at ambient temperature or overnight incubation at 4°C, cultures were rinsed and incubated in species-specific AF488 or AF594-conjugated secondary antibodies followed by Hoechst 33258 (0.5 µg/ml; Sigma) to counterstain nuclei. Cells were imaged using Nikon/Leica microscopes and quantified using MetaMorph Offline software (Molecular Devices).

Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and RNA (1 µg) was first DNase treated and then reverse transcribed to cDNA with oligo(dT) using the Promega Reverse Transcriptase System. Reactions were performed in three technical replicates using SYBR Green master mix (Applied Biosystems) using specific primer sequences (Table 2). Each PCR cycle consisted of 95°C for 10 min, 95°C 30 s → 58°C for 60 s, for 50 cycles, and 72°C 5 min. The melting curve was measured and recorded from 65°C to 95°C in increments of 0.05°C to 0.5°C. Genes of interest were normalized to RPL13 ribosomal Protein L13A, and calculated by 2^{-CT} method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Cell preparation for transplantation

Cells were incubated in TrypLE (Life Technologies), rinsed with DMEM, incubated in DNase/DMEM (50%/50%), rinsed and dissociated with fire polished glass Pasteur pipette into a single cell suspension. Cells were passed through a 30 µm filter (Miltenyi Biotec, Auburn, CA, USA), centrifuged (1000 RPM, 5 min) and resuspended in hibernate media (2.2g/L KCl, 0.9 g/L Glucose, 0.05g/L MgCl₂.6H₂O, 1.51 g/L NaH₂PO₄.H₂O, 0.89 g/L Na₂HPO₄.2H₂O, 0.2% Lactic Acid, pH7.2 with KOH Pellets, 25.5 g/L Sorbitol) at the appropriate cell concentration and kept on ice for transplantation.

Cell transplantation

All animal procedures were carried out in accordance with the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee under protocol #3133 (Neural Stem Cells for Amyotrophic Lateral Sclerosis) and National Institutes of Health standards of animal care. Athymic nude rats (Hsd:RH-Foxn1^{tmu}; Harlan Laboratories) were transplanted with 2 µl of fNPCs, iNPCs^{AD} or iNPCs^{SU} in 3 distinct sites, 1 mm apart at a concentration of 20,000 cells/µl, 60,000 cells/µl or 100,000 cells/µl. Briefly, rats were anesthetized with isofluorane and transferred to a stereotaxic frame (David Kopf instruments, Tujunga, CA, USA). The 12th rib of the rat was identified and an incision was performed in the skin and muscle to expose the lumbar vertebrae. A hemi-laminectomy was performed on the side of the surgery to expose the spinal cord and dura was cut. Cells were loaded into a 45° beveled glass micropipette connected to a 10 µl Hamilton syringe and a microinjection pump. Cells were then injected directly into the parenchyma (0.8 mm medio-lateral, 1.8 mm dorso-ventral) at a rate of 1 µl/minute.

Tissue collection and histology

Rats were anesthetized and transcardially perfused with 0.9% NaCl and fixed with 4% PFA (EMS 1224SK-SP). Tissues were collected and post-fixed overnight in 4% PFA and transferred into 30% sucrose for 48 hours prior to sectioning (35 µm) on a sliding microtome. Prior to sectioning, the side contralateral to surgery was identified by notching the dorsal horn. Every 1/12th sample through the lumbar spinal cord was immuno-stained with the following antibodies: STEM101, STEM121, STEM123, Nestin, Ki67, and Aquaprin-4 (AQP4) (Table 1) according to standard techniques. Sections were stained with

the Alexa-488 or Alexa-594 coupled secondary antibodies (Life Technologies) and nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Life Technologies).

Stereology for immunohistological quantifications

Stereological quantifications of STEM 101 and Ki67 were performed using the optical fractionator method from StereoInvestigator software (MBF Biosciences) associated with an Axio Imager M2 microscope (Zeiss) at 60 $\times$ magnification. Stereological analysis was performed at 1/12th sample interval for STEM101 and Ki67 cell counts and the entire ipsilateral side of the spinal cord sections within the grafted area that was traced. The counting parameters were distance between counting frames (500 μ m); counting frame size (75 μ m $\times$ 75 μ m); dissector height (24 μ m) and the guard zone (2 μ m). The range of coefficient of error (CE, Gundersen $m=1$) and number of markers counted are reported in figures associated with stereological counts. Overall, the lowest number of markers counted and highest CE were observed in the data obtained from iNPC^{AD} cultures and associated with the overall small size and localized nature of the surviving graft. To assess absolute cell migration, the total number of sections (out of 1/12th series) containing STEM101+ cells were counted and multiplied by a factor of 12 (series) and by the section thickness (35 μ m).

Statistical analysis

Prism software (GraphPad software, La Jolla, CA) was used for statistical analyses. Immunocyto/histochemical analyses and cell survival quantification were expressed as mean values $\pm$ SEM and analyzed by two-tailed *t*-test or two-way ANOVA with Bonferroni *post hoc* test. Differences were considered significant when $p < 0.05$.

RESULTS

Neural stem cell spheres are generated from human iPSCs

Skin fibroblasts were reprogrammed from multiple healthy individuals to iPSCs using a non-integrating system based on the oriP/EBNA1 (Epstein-Barr nuclear antigen-1). This episomal plasmid vector system was used to avoid potential deleterious effects of proviral sequences randomly inserted into the genome (Okita et al., 2011a; Sareen et al., 2012; Yu et al., 2009). Multiple clonal iPSC lines were generated showing typical PSC-like morphology with a high nuclear-cytoplasmic ratio (Sareen et al., 2012), alkaline phosphatase activity and surface and nuclear expression of pluripotency markers, SSEA4, TRA-1-81, TRA-1-60, OCT3/4, SOX2, NANOG (Fig. 1A). An iPSC line from one individual (00iCTR-n1) was picked for further in-depth studies and characterized using a battery of pluripotency assays. The iPSC population maintained greater than 80% OCT3/4 and SSEA4 double positive cells (Fig. 1B) and a normal karyotype over many passages (Fig. 1C). PluriTest analysis, a validated open-access bioinformatics assay for assessing pluripotency using transcriptome profiling, showed that the 00iCTR line had a high pluripotency score and low novelty score, similar to other PSC lines and in contrast to differentiated fibroblasts and NPCs (Fig. 1D). Importantly, qRT-PCR and Southern blot analyses confirmed that the 00iCTR line lacked expression of exogenous transgenes, demonstrating that the oriP/EBNA1 plasmid-based method generated “footprint-free” iPSC lines (Fig. 1E and F).

We have recently developed an easy ("EZ") and robust method to generate multipotent neural stem cells (NSCs) from human iPSCs without utilizing embryoid body formation, which we termed EZ spheres (Ebert et al., 2013). We used the same approach here where iPSC colonies were lifted from feeder-free Matrigel and cultured in suspension medium containing high concentrations of EGF (100 ng/ml) and FGF2 (100 ng/ml) supplemented with heparin. Spherical aggregates of pre-rosette NSCs formed within 2 weeks and could be expanded as EZ spheres for over 40 weeks in culture (Fig. 1G).

Human iPSC-derived EZ spheres can be differentiated into neural progenitor cells (iNPCs)

We previously published that EZ spheres are a very primitive type of neural stem cell at a stage prior to early neural tube (rosette) formation (Ebert et al., 2013) and that such cells do not engraft well into the spinal cord (Fig. 6 and other unpublished data). In order to generate a cell type more similar to neural progenitor cells isolated from fetal tissues we directed EZ spheres towards a caudalized spinal cord fate by withdrawing EGF and FGF2 and applying retinoic acid for 11 days, a morphogen known to drive hindbrain and spinal cord development (Li et al., 2005; Novitsch et al., 2003; Pierani et al., 1999; Sockanathan and Jessell, 1998; Wichterle et al., 2002). Mitogen withdrawal and retinoic acid addition was used with two different approaches shown in Fig. 2A. Either iNPCs were cultured on Matrigel substrate as an adherent monolayer (iNPCs^{AD}) or cultured as aggregates in suspension (iNPCs^{SU}). Following caudalization in retinoic acid, iNPC monolayers and spheres were re-introduced into high EGF and FGF-2 mitogens for subsequent expansion.

The iNPCs^{AD} cultures could only be expanded enzymatically for 12–15 weeks, while the iNPCs^{SU} expanded using the chopping technique (similar to fNPCs) maintained proliferative capacity without senescing for 26–30 weeks and a stable normal karyotype at different passages (Figure 2B). The iNPCs^{SU} showed many pseudopodia or cilium-like structures (Figure 2C-C2), as has also been observed with fNPCs (Lobo et al., 2003; Svendsen et al., 1998). The iNPCs^{AD} were healthy following plate-down from EZ spheres (Fig. 2D) and during retinoic acid patterning Fig. 2E). Upon reintroduction of mitogens for expansion, their morphological features on Matrigel substrate appeared similar to adherent neural stem and progenitor cells in other studies (Koch et al., 2009; Tamaki et al., 2002; Taupin, 2006) (Fig. 2F). After over 10 weeks in culture they adopted a more mature cellular morphology with larger cell size and visible cytoskeletal structures (Fig. 2G). Mitogen withdrawal and ciliary neurotropic factor (CNTF) addition could differentiate the iNPCs^{AD} into cell types with a typical mature astrocyte morphology and processes (Fig. 2H), comparable to protoplasmic astrocytes reported to possess dense ramifications and 'spongiform' morphology (Bushong et al., 2004).

Ultimately, both cultures displayed senescence following continual passaging suggesting that these cells are more similar to fNPCs (Ostenfeld et al., 2000; Wright et al., 2006) rather than immortal PSCs (Takahashi et al., 2007; Thomson et al., 1998). We have previously shown that early senescence of fNPCs can be avoided by adding leukemia inhibitory factor (LIF) to the cultures (Wright et al., 2003; Wright et al., 2006). In order to establish if iNPCs could also respond to LIF, we first confirmed that both suspension and adherent iNPCs expressed LIF receptors (Fig. 2I). Though the LIF receptor was expressed, it appeared that

LIF addition did not change the senescence pattern observed for cultures of either the iNPCs^{AD} or iNPCs^{SU}, ultimately losing the pseudopodia-like structures (Fig. 2J). This interesting difference in response to LIF suggests that while some aspects of the iNPC and fNPC profiles are similar, they are certainly not identical.

Molecular and cellular characterization of iNPCs reveals a predominant astroglial progenitor composition

As the plated iNPCs^{AD} appeared morphologically like astrocytes, we wanted to further establish how similar these cultures were to neural progenitors or astroglia. To do this, we assessed gene expression of multiple astrocyte markers, Aquaporin 4 (*AQP4*), glial-fibrillary associated protein (*GFAP*), glial high affinity glutamate transporter (*GLAST/SLC1A3 / EAAT-1*), *S100B*, and functional astroglia genes *SLC26A7* (Anion exchange transporter/ Solute Carrier Family 26, Member 7) and *SLC38A1* (Sodium-coupled neutral amino acid transporter 1/Solute Carrier Family 38, Member 1) (Krencik et al., 2011). Analysis by qRT-PCR showed iNPCs^{AD} had increased levels of *GFAP* mRNA compared to iPSC and EZ spheres, however, the other markers were expressed in similar levels to EZ spheres. Interestingly, expression of *AQP4*, the primary water conductance transport protein in astrocytes, was not detected in iNPCs^{AD} (Fig. 3A). In contrast, both iNPCs^{SU} and fNPCs showed a clear up-regulation of these astroglia genes when compared to iPSCs and EZ spheres (Fig. 3A).

In order to assess the cell types and their morphology present within the NPC cultures, we plated cells onto Matrigel coated coverslips for 24hrs and used immunocytochemistry to detect the neural progenitor and astrocyte markers nestin, *GFAP*, *S100B*, *Aldh1L1*, the pluripotency marker *OCT3/4*, and the neuronal marker *TuJ1*. As shown previously by Ebert et al. (2013), cells plated directly from EZ spheres mainly expressed nestin and *GFAP*, with minimal *S100B*, *TuJ1* or *OCT3/4* staining (Fig. 3B–F). Qualitatively there was low expression of *A2B5* (Fig. 3C, G, K and P) and *Aldh1L1* (Fig. 3D, H, L Q) in iNPCs and fNPCs when compared to the EZ spheres. The iNPCs^{AD} showed an increase in nestin, however, no increase was observed for *GFAP* and *S100B* protein levels compared to EZ spheres (Fig. 3E and I), which is in contrast to the increased *GFAP* mRNA expression (Fig. 3A). Both iNPCs^{SU} and fNPCs showed an increase in nestin (Fig. 3K and P), *GFAP* and *S100B* (Fig. 3M and R) expression compared to EZ spheres (Fig. 3B, C and E). In addition to this similar protein expression profile for iNPCs^{SU} and fNPCs, the morphology of astroglia from iNPCs^{SU} and fNPCs resembled each other closer, while the astroglia from iNPCs^{AD} displayed a more spindly morphology. None of the cultures expressed *OCT3/4* positive cells (Fig. 3F, J, N and S) and very few stained for *TuJ1* positive neurons (Fig. 3B, F, J, N and S). Together these data show that both iNPCs^{AD} and iNPCs^{SU} have a predominately astroglial progenitor composition, though the molecular and morphological astroglial characteristics of iNPCs^{SU} are more akin to fNPCs.

Since our goal was to obtain transplantable iNPCs with a CNS caudalized/posterior regional identity, we determined whether our protocol for generating iNPCs^{SU} led to specification of rostral-caudal, hindbrain/spinal cord, and/or ventral fates by examining distinct expression of region-specific genes, including caudal homeodomain transcription factors, *HOXB3* and

HOXB4. Differential treatment of iPSC-derived EZ spheres with patterning morphogens promoting different CNS-specific regional fates including, caudal/posterior (retinoic acid), ventral (purmorphamine (PMN); Shh agonist) and rostral/anterior (Dkkopf1 (Dkk1); WNT signaling antagonist) generates different iNPCs^{SU} with distinct expression of region-specific genes confirmed by qRT-PCR gene expression analysis (Fig. 3T). The iNPCs^{SU} produced using retinoic acid, iNPCs^{SU} (RA), significantly up-regulate genes specific to hindbrain/spinal cord (*KROX20*, *ISL1*) and caudal (*HOXB4*, *HOXB3*, *SPARCL1*, *NTRK2*) identities. In contrast, iNPCs^{SU} produced by patterning EZ spheres with DKK1 and PMN, iNPCs^{SU} (DKK1+PMN), up-regulate gene expression specific to rostral (*DLX1*, *FOXG1*, *SIX3*) and ventral (*NKX2.1*) identities, and lack signatures of spinal cord and caudal gene expression (Fig. 3T).

Differentiation of iNPCs leads to maturation of astroglia

To determine phenotypes upon differentiation, we assessed astroglial progenitor and mature astrocyte marker profile of iPSC-derived neural stem and progenitor cells (EZ spheres and iNPCs) to fNPCs by plating them on laminin/matrigel coated coverslips in mitogen-free media (withdrawal of EGF and FGF2) for 7 days. We observed that astroglial progenitor markers (A2B5, Aldh1L1) were down-regulated in differentiated iNPCs^{AD} (Fig. 4E, F), iNPCs^{SU} (Fig. 4I, J) and fNPCs (Fig. 4M, N) when compared to EZ spheres (Fig. 4A, B). The developmental astrocyte markers, nestin and GFAP, and mature marker, S100B, were strongly expressed in both iNPCs^{SU} and fNPCs (Fig. 4K, O) when compared to EZ spheres and iNPCs^{AD} (Fig. 4C and G). The pluripotency marker was undetectable and the TuJ1 pan-neuronal marker was at similar levels in all cell types (Fig. 4D, H, L, and P). Significantly, differentiation led to robust expression of mature and functional astrocyte genes, AQP4 and GLAST (SLC1A3/EAAT-1), which was similar between iNPCs^{SU} and fNPCs (Fig. 4Q, R, T and U). Both iNPCs^{SU} and fNPCs contained proliferating populations of cells, 29±2% and 36±4%, respectively, as determined by Ki67 marker staining and counts (Fig. 4S and V).

Transcriptome profiling shows that suspension culture iNPCs^{SU} and human fNPCs are similar

We further investigated the relationship between all the fNPCs and iNPCs (adherent and suspension) by whole transcriptome analysis and bioinformatics testing based on a pluripotency characterization assay, known as PluriTest at www.pluritest.org (Muller et al., 2011). In this test human microarrays are performed and mRNA expression values of all probes including pluripotency-associated genes are scored against samples in the stem cell model matrix, consisting of 264 pluripotent cell lines (223 hESC and 41 human iPSC) and 204 samples derived from somatic cells and tissues. A “novelty” score is generated based on overall gene expression patterns of well-characterized pluripotent samples against the sample of interest. A low novelty score indicates that the test sample can be well reconstructed based on existing data from other well-characterized iPSC and ESC lines. A high novelty score indicates that there are patterns in the tested sample that are not concordant with gene expression patterns of PSC, and instead of more variant cell types, thus making them “novel”. As expected, iPSC lines, including 001iCTR, expressed few novel genes and mostly pluripotent genes leading to a score of < 1.6 in this test (Fig. 5A, Green bars). In contrast, EZ spheres, iNPCs^{SU} and ^{AD}, and fNPCs had higher novelty scores

(Fig. 5A, Red bars). Another way to plot the gene expression data is to rank pluripotency based on a positive score index. Samples with positive values are more similar to the pluripotent samples in the model matrix than to all other classes of samples in the matrix. The area between the red lines indicates the range that contains ~95 percent of the pluripotent samples tested, while the blue lines indicate those scores that were observed in ~95 percent of the non-pluripotent samples (Fig. 5B). Again iPSCs, including 001iCTR, were between the red dashed lines, whereas all NPCs scored below the blue dashed lines. Interestingly, EZ spheres were found to maintain a pluripotency score (-51.8) higher and closer to PSCs than any non-PSC sample tested thus falling in-between PSCs and more differentiated somatic cells, further supporting their pre-rosette NSC status (Ebert et al, 2013). Both the fNPCs (-93.9) and iNPCs^{SU} (-82.7) had similar scores much lower on this scale as did their differentiated counterparts, 00iNPCs^{SU} Df. and fNPCs Df. (Fig. 5B). The combination of the two scores provides a more comprehensive perspective of this data by combining the pluripotency score on the y-axis and the novelty score on the x-axis. The red and blue background show the empirical distribution of the pluripotent (red) and non-pluripotent (blue) samples in the stem cell model matrix. Using this analysis, it confirmed that the iNPCs^{SU} and fNPCs were similar to each other, while the iNPCs^{AD} were further away (Fig. 5C). The EZ spheres were not pluripotent, but remained closer to the iPSCs (Fig. 5C). This paradigm was also validated by hierarchical clustering analysis that grouped the fNPCs and iNPCs^{SU} together (Fig. 5D).

Next, we utilized whole-genome transcriptomics profiling and scatter plot analysis to compare iNPCs^{SU} with EZ spheres (Fig. 5E), iNPCs^{AD} (Fig. 5F), and fNPCs (Fig. 5G). When the differentiated iNPCs^{SU} were also compared with differentiated fNPCs, their profiles coincided significantly (Fig. 5H). The iNPCs^{SU} had acquired different transcription profiles making them more distant from EZ spheres and iNPCs^{AD} (Fig. 5E and F) and rather much closer in gene expression patterns to fNPCs (Fig. 5G), further supporting the idea that they were being patterned towards a human fNPC phenotype. In this array data the astroglia-related genes, *AQP4*, *S100B*, *SLC1A2*, *NTRK2*, and *SLC1A3*, were found to be highly expressed in iNPCs^{SU} compared to iNPCs^{AD} (Fig. 5F), confirming previous observations (Fig. 3A).

Suspension culture iNPCs^{SU} exhibit greater survival in rat spinal cords

Prior to transplantation, the dissociation of NPC sphere cultures may compromise cell viability. To address this, we assessed cell death and integration parameters for identical batches of NPCs transplanted into athymic rat spinal cords. For *in vitro* evaluation, the NPCs used for transplantation studies were plated and differentiated for 7 days. Immunostaining with FITC conjugated Annexin V, an early cell death marker, did not show any cytotoxicity (Fig. 6A and B), and quantification demonstrated >98% cell viability (data not shown). Further, pyknotic or condensed apoptotic nuclei were undetectable by nuclear Hoechst dye labeling (Fig. 6A and B), a previously validated cell death assay (Sareen et al., 2006). Instead, robust GFAP+astrocyte differentiation and cell survival were observed in both NPC cultures (Fig. 6A and B). In addition, spinal cord sections from animals transplanted with the same batch of NPCs were analyzed using a well-known functional astrocyte marker (AQP4) and human-specific cytoplasmic antibody (STEM121). Careful

examination by confocal microscopy confirmed that the transplanted human iNPCs^{SU} and fNPCs can express the functional AQP4 astroglial marker *in vivo* and can anatomically integrate in the rodent spinal cord also (Fig. 6C and D).

To next establish whether the iNPCs behaved in a similar way to fNPCs *in vivo*, we transplanted EZ spheres, iNPCs^{AD}, iNPCs^{SU} and fNPCs into the spinal cord of athymic nude rats at three different cell doses (20,000, 60,000 and 100,000) (Fig. 7). At three weeks after transplantation there were clear differences in cell survival and distribution. All graft types labeled with STEM101 for human-specific nuclear staining and STEM123 for human-specific GFAP staining and expressed nestin. EZ spheres (Fig. 7A–D) and iNPCs^{AD} (Fig. 7F–J) showed much poorer graft survival compared to iNPCs^{SU} (Figs. 7K–O). The iNPCs^{SU} also showed robust survival comparable to fNPC grafts (Figs. 7P–T). These observations were confirmed by stereological counts showing that cell survival of iNPCs^{SU} was significantly greater compared to iNPCs^{AD} and similar to (or slightly greater than) that seen with fNPC transplants (Fig. 7U). Indeed, the significantly greater cell survival of iNPCs^{SU} compared to iNPCs^{AD} was maintained at each transplant dose (Fig. 7V). Interestingly, the numbers of proliferating cells detected at the time of sacrifice using the proliferative marker Ki67 was not different between the three groups (Fig. 7W). This suggests that the increased number of cells was a result of increased survival rather than proliferation, though, we cannot rule out early proliferation of the cells prior to sacrifice. There was no significant difference in cell migration as the iNPCs^{SU} appeared similar to fNPCs (Fig. 7X).

DISCUSSION

Neural stem and progenitor cell transplantation holds great potential for providing cell replacement and support to the brain or spinal cord afflicted by neurodegenerative disorders or injury. Fetal-derived neural progenitor cells (fNPCs) grown either as aggregates or monolayer cultures have been around for many years, can be successfully propagated and have advanced into human clinical trials (Neuralstem Inc. and Emory University, 2011; StemCells, 2006; StemCells, 2011; StemCells, 2012; Taupin, 2006). However, they are limited by ethical and cell expansion considerations. By using EZ spheres (Ebert et al., 2013) as the starting source of material we found that both adherent and suspension NPC cultures could be generated from immortal iPSCs that could be proliferated for extensive periods. Therefore, this study provides a much-needed simple protocol for the generation of a stable, easily maintained, highly expandable and bankable population of human iPSC-derived NPCs (iNPCs) and describes their properties in culture and upon transplantation.

To generate iNPCs with ease and in abundance, we avoided embryoid body formation and instead used EZ spheres, a stable source of pre-rosette multipotent stem cell spheres generated from human iPSCs (Ebert et al., 2013). The EZ spheres were directed through caudal patterning using all-trans retinoic acid to produce iNPCs, which were then expandable after addition of EGF and FGF-2 mitogens. The iNPCs were maintained either as adherent cultures (iNPCs^{AD}) that were passaged enzymatically for up to 15 weeks past EZ sphere stage or as suspension cultures (iNPCs^{SU}) that were passaged for up to 30 weeks using a chopping technique we have described previously for fNPCs (Svendsen et al., 1998) and EZ spheres (Ebert et al., 2013). An interesting difference found in culture was related to

chromosomal abnormalities and aneuploidies which occur stochastically in ES and iPS cell cultures (trisomy 12, 17, and X) and in various fNPC (trisomy 7 and 19) lines (Sareen et al., 2009), but were not detected in the iNPC line even after extensive culturing. Following reprogramming, none of the episomal plasmid genes were still expressed, which importantly demonstrated that the genome is free from potentially deleterious exogenous transgenes. This fact, along with the stable karyotype overtime, makes these iPSC-derived NPCs reliable for researchers and potentially safe for future clinical trials.

A number of different culture protocols exist for the generation of PSC-derived neural progenitor cells, including a complex four-stage culture system involving RA-mediated induction (Yuan et al., 2013), stromal cell co-culture, adherent culture in EGF and FGF-2 (Koch et al., 2009; Sun et al., 2008), conditioned and serum-free medium induction (Daadi et al., 2008), and neural rosette isolation. However, these methods can be lengthy, laborious, and costly, provide low yields, and none of these studies have reliably shown good cell engraftment capacity in the spinal cord. Retinoic acid has been demonstrated to promote human ESC differentiation into neural stem cells, but efficiency was low and the survival capacity was limited (Baharvand et al., 2007; Bain et al., 1995; Fraichard et al., 1995; Guan et al., 2001; Rohwedel et al., 1999; Strubing et al., 1995; Zhou et al., 2008). In contrast, only a few hESC or hiPSC protocols have been described that produce neural progenitor cells capable of generating astrocytes, and the most thorough method required over 100 days of differentiation (Krencik et al., 2011; Krencik and Zhang, 2011).

Astrocytes comprise the majority of the brain, play a prominent role in protection through glutamate uptake and are known to be defective in some neurological disorders, like ALS. Providing a population of cells that can easily generate astrocytes would facilitate both researchers interested in further understanding the role of astrocytes and clinicians wanting to assess their benefits in trials. Here we investigated human induced pluripotent stem cells (iPSCs) as a promising alternative to provide NPCs that would be abundantly available and then go on to produce astrocytes following transplantation. Upon differentiation in culture, both iNPCs^{AD} and iNPCs^{SU} showed a propensity towards an astroglial lineage, with iNPCs^{SU} being more propitious and resembling a differentiation pattern similar to what was previously reported for fNPCs. In addition, iNPCs^{SU} could be regionalized to caudal spinal cord fate with retinoic acid and also shared similar transcriptomes and gene expression profiles with fNPCs. The propensity towards an astroglial lineage makes these iNPCs a promising choice for transplantation trials needing astrocytes.

The transplantation of neural progenitors that can mature into astrocytes in the spinal cord is being pursued as a novel treatment for ALS where the astrocytes may protect dying motor neurons (Lepore et al., 2011; Neuralstem Inc. and Emory University, 2011; Suzuki et al., 2007; Suzuki and Svendsen, 2008). Here we show that iNPCs survived in the spinal cord of athymic nude rats, with expression of mainly nestin after 1 week and GFAP after 3 weeks. These results show that grafted iNPCs survive well in the spinal cord and have the potential to differentiate into astrocytes. However, 3 weeks is likely insufficient for complete differentiation, as we have previously shown with fNPCs it may take up to 3 months to completely differentiate into GFAP-positive astrocytes in large numbers (Ostenfeld et al., 2000). Interestingly, we found that the iNPCs^{SU} and fNPCs grown as free-floating spheres

survive and integrate better following transplantation than the iNPCs^{AD}, grown as a monolayer, suggesting that maintaining a three-dimensional cellular aggregate niche may provide better culture conditions for long-term survival and cell engraftment in the host. Importantly, both human iNPCs^{SU} and fNPCs also express GFAP and another putative astroglial marker Aquaporin 4 (a water channel protein) *in vivo* post-transplantation, suggesting that they mature to astroglia and anatomically integrate in the rodent spinal cord. In contrast, three-dimensional EZ sphere suspension cultures did not survive well in the spinal cord suggesting that differentiation towards a neural progenitor phenotype is required to optimize transplant survival. Overall these data suggest iNPCs may be an ideal candidate for transplantation, in particular for diseases such as ALS where cell therapy is being actively pursued (Gowing and Svendsen, 2011).

Clearly we were concerned with possible tumor formation from remaining pluripotent cells within the iNPC cultures as this has been an ongoing concern for the field (Blum and Benvenisty, 2009; Bonnamain et al., 2012; Ghosh et al., 2011; Kawai et al., 2010; Zhang et al., 2012; Zhang et al., 2011). However, in no case following iNPC transplantation did we find any histopathological evidence of increased cell proliferation and tumor forming capacities. One possible reason for this is because prior to transplantation the pluripotent cells were first converted to EZ spheres and cultured for greater than 10 weeks and then subsequently driven to iNPC cultures for a further 10 weeks. However, three weeks is certainly not long enough to fully assess potential tumor development, and hence long-term testing of iNPC safety profile is still required.

In summary, we demonstrated that human iPSCs can be used to efficiently derive NPCs that are easy to culture and bank, have an astroglial differentiation propensity and survive transplantation. These characteristics, along with their transcriptome profiles, pluripotency and novelty scores, are all very similar to the human fetal-derived NPCs that our lab has extensively characterized (Ostenfeld et al., 2000; Svendsen et al., 1998; Wright et al., 2003; Wright et al., 2006). In addition to avoiding the ethical issues of using human fetal tissues, iPSC-derived NPCs permit the future possibility of autologous transplantation thus avoiding complex immune suppression problems that currently plague neural transplantation therapies. As such, human iPSC-derived NPCs are a novel and promising resource for cell-based therapies aiming to replace cells or to modulate the environment for host neuron protection or regeneration in injury and disease.

Acknowledgments

The authors acknowledge Dr. Soshana Svendsen for critical review and editing of the manuscript.

FUNDING: This work was supported by Cedars-Sinai Institutional startup funds (CNS), California Institute for Regenerative Medicine grants, RT-02040 (CNS, DS) and DR2A-05320 (CNS). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Figure 1. Human iPSC and EZ sphere generation

(A) Bright-field images of the reprogrammed iPSC colonies from healthy subject fibroblasts maintained on Matrigel/mTeSR1 show high nuclear-to-cytoplasmic ratio, typical of standard pluripotent stem cells (hESCs and hiPSCs). Successfully reprogrammed iPSC lines show positive staining for alkaline phosphatase. Immunostaining shows expression of embryonic and pluripotency stem cell surface antigens, SSEA4, TRA-1-60, TRA-1-81; and nuclear pluripotency markers OCT3/4, SOX2 and NANOG. (B) Flow cytometry analysis scatter plot shows that 00iCTR maintained SSEA4 and OCT3/4 double positive pluripotent cell population > 80% over long-term passaging. (C) The 00iCTR line maintained a normal G-band karyotype. (D) Transcriptomics and bioinformatics based PluriTest characterization (Muller et al., 2011) confirmed pluripotency of 00iCTR. Chart combines pluripotency score on y-axis and novelty score on x-axis. The red and blue background hint to the empirical distribution of the pluripotent (red) and non-pluripotent (blue) samples in test data set

(Muller et al., 2011). **(E)** Quantitative RT-PCR analyses of *POU5F1* (*OCT4*), *KLF4*, *SOX2*, *LIN28*, *L-MYC*, and *EBNA-1* expression in 00iCTR iPSCs relative to H9 hESCs. “CDS” indicates that primers designed for the coding sequence measured expression of the total endogenous gene expression only, whereas “Pla” indicates that primers designed for the plasmid transgene expression, including *EBNA-1* from plasmid backbone, were not detected. Data are represented as mean $\pm$ SEM. **(F)** Southern blot analysis of genomic DNA from 00iCTR with a plasmid back-bone specific probe common to the reprogramming plasmids used shows lack of exogenous transgene-integration. Negative control: H9 hESC line. Positive controls: Other integrating iPSC lines generated using the episomal plasmid technique showing plasmid-based gene integration. Technical control: A single copy of the plasmid DNA can be detected using this technique. **(G)** Schematic depicting EZ sphere generation from 00iCTR iPSCs. Representative data and images for iPSC and EZ sphere generation here are depicted from a healthy individual iPSC line (00iCTR) with similar results for other iPSC lines.

Figure 2. Generation of iPSC-derived neural progenitor cells (iNPCs)

(A) Schematic illustrating iNPC generation from iPSCs via EZ spheres formation using an adherent ($iNPCs^{AD}$) and a suspension ($iNPCs^{SU}$) culture protocols. (B) The 00iCTR-derived $iNPCs^{SU}$ line subjected to G-Band karyotyping at multiple passages (p) 8 and 25 stably maintained a normal male 46, XY karyotype. (C) Light microscopy image of a representative $iNPCs^{SU}$ sphere in suspension. Black box in (C) shows a region on the sphere magnified in the right adjacent image (C1). Black box in (C1) shows a representative region on the sphere with pseudopodia extensions protruding from an $iNPCs^{SU}$ sphere and magnified in the right adjacent image (C2). Black arrows in (C2) point to the pseudopodia extensions of the $iNPCs^{SU}$. (D–H) Light microscopy images depicting temporal progression of $iNPCs^{AD}$ formation from (D) EZ spheres dissociated and plated on Matrigel at p16, (E)

after all-trans retinoic acid (ATRA) addition for 11 days, **(F)** removal of ATRA, re-introduction of EGF+FGF2, iNPCs^{AD} formation, and culture at p5, and **(G)** at p12 after which they senesce. **(H)** Morphology of mature astrocytes generated from iNPCs^{AD} after differentiation following mitogen withdrawal and CNTF addition showing more complex astroglial processes and ramifications. **(I and J)** Effect of LIF on iNPCs^{SU}, **(I)** RT-PCR for iNPCs^{AD}, iNPCs^{SU}, and EZ spheres show strong gene expression of LIF receptors, *gp130* and *LIFRβ* in iNPC^{SU} cultures. **(J)** Light microscopy image of representative iNPC^{SU} spheres after LIF addition to EGF+FGF2 cultures or EGF cultures. Both cultures senesced rapidly after LIF addition and lost the pseudopodia extensions of iNPC^{SU} spheres maintained in EGF+FGF2 media shown in Figure 2C-C2. Scale bars: **C** = 50 μm, **C1–C2** = 10 μm, **D–H, and J** = 50 μm. Results are representative of at least one of three independent biological samples with similar results.

Figure 3. Phenotype characterization of iNPCs

(A) Quantitative RT-PCR characterization of known astroglia developmental and functional genes, *AQP4*, *GFAP*, *GLAST* (*SLC1A3/EAAT-1*), *S100B*, *SLC26A7* and *SLC38A1* showed strong expression of these genes in iNPCs^{SU} as in fNPCs. *AQP4*, *S100B*, *GLAST*, and *SLC26A7* were expressed at significantly greater levels ($10^1 - 10^4$ fold) in iNPCs^{SU} vs. iNPCs^{AD}. Error bars are mean $\pm$ SEM of three technical replicates. (B) Immunocytochemical staining cell counts of nestin, GFAP, S100B and TuJ1 (β_{III} -tubulin) positive cells following short-term (24 hours) plate-down of EZ spheres, iNPCs^{SU}, iNPCs^{AD}

and fNPCs. There were significantly more S100B+ cells in iNPCs^{SU} and fNPCs vs. iNPCs^{AD} or EZ spheres. ** = $p < 0.01$; * = $p < 0.05$; two-way ANOVA (Bonferroni post-test). Error bars are mean $\pm$ SEM of three biological replicates. Representative immunostaining images of (C–F) EZ spheres, (G–J) iNPCs^{AD}, (K–N) iNPCs^{SU}, and (P–S) fNPCs, using protein markers for astroglial progenitors, (C, G, K, P) A2B5 and nestin, (D, H, L, Q) ADH1L1 and GFAP; differentiated astroglia, (E, I, M, R) S100B and GFAP; (F, J, N, S) pan-neurons, Tuj1 and pluripotency, OCT3/4. Scale bars = 100 μ m. (T) Determination of regional identity (rostro-caudal, hindbrain/spinal cord, ventral) of iNPCs^{SU}. CNS patterning morphogens promoting caudal/posterior (RA), ventral (PMN), and rostral/anterior (Dkk1) fates generated iNPCs^{SU} with distinct expression of region-specific genes as shown in qRT-PCR expression histogram plots. The iNPCs^{SU} generated with RA patterning, iNPCs^{SU} (RA) (in black bars), expressed hindbrain/spinal cord (*KROX20*, *ISL1*) and caudal (*HOXB4*, *HOXB3*, *SPARCL1*, *NTRK2*) genes. The iNPCs^{SU} (DKK1+PMN) made with DKK1 and PMN (in blue bars), expressed genes of rostral (*DLX1*, *FOXG1*, *SIX3*) and ventral (*NKX2.1*) identities, and lacked spinal cord or caudal genes. Experiments on all cell types were performed at similar passage numbers (between p8–25) using independent biological samples in at least three replicates. Results are mean $\pm$ SEM from representative experiments.

Figure 4. Differentiation of iNPCs into astrocytes

Differentiation phenotypes of EZ spheres, iNPCs and fNPCs were compared by immunostaining for astroglial and cell proliferation markers at similar passage numbers (between p8–25) following dissociation, plate-down and differentiation by mitogen withdrawal (EGF and FGF2) for 7 days. Astroglial progenitor (A2B5, Aldh1L1) markers were down-regulated in differentiated (E–F) iNPCs^{AD}, (I–J) iNPCs^{SU}, and (M–N) fNPCs when compared to (A–B) EZ spheres, while astrocyte developmental (nestin, GFAP) and mature markers (S100B) were robustly and equivalently expressed in (K) iNPCs^{SU} and (O)

fNPCs when compared to **(G)** iNPCs^{AD} and **(C)** EZ spheres. **(D, H, L, and P)** OCT3/4 (pluripotency) protein was undetectable and TuJ1 (pan-neurons) protein was at similar levels in all cell types examined. **(Q, R, T, and U)** Functional astrocyte genes, AQP4 and GLAST were actively expressed at similar levels between iNPCs^{SU} and fNPCs. **(S, V)** Human iNPCs^{SU} (29%) and fNPCs (36%) maintained cell proliferation rates as determined by Ki67 staining and counting. Representative images are depicted from at least three independent biological replicates with similar results.

Figure 5. Transcriptome profiling of iNPCs

(A) Novelty score graph: A score based in well-characterized pluripotent samples in the stem-cell model matrix. Samples are color coded, green (pluripotent) and red (not pluripotent or closer to somatic). All iPSC-derived neural derivatives, including EZ spheres, iNPCs^{SU}, iNPCs^{AD}, and fNPCs (red bars) were more dissimilar to the pluripotent samples in the model matrix than other pluripotent samples (green bars). EZ spheres were the least novel from all neural derivatives tested. (B) The Pluripotency score giving an indication if a sample contains a pluripotent signature above the red-dotted line shows that all iPSC-derived neural derivatives were not pluripotent. The EZ spheres possessed the most and iNPCs^{AD} second-most pluripotent signature closer to PSCs than iNPCs^{SU} and other NPC derivatives. (C) Chart combines pluripotency score on y-axis and novelty score on x-axis. The red and blue background hint to the empirical distribution of the pluripotent (red) and non-pluripotent (blue) samples. The iNPCs^{SU} were closer to fNPCs and other somatic cells (blue cloud) in the PluriTest test dataset. (D) Hierarchical clustering of the samples based on

PluriTest Gene Expression profile shows that fNPCs and iNPCs^{SU} clustered close to each other. Whole-transcriptome gene expression scatter plots of **(E)** EZ spheres vs. iNPCs^{SU}, **(F)** iNPCs^{AD} vs. iNPCs^{SU}, **(G)** fNPCs vs. iNPCs^{AD}, and **(H)** differentiated fNPCs vs. differentiated iNPCs^{SU} showed that even though EZ spheres, iNPCs^{AD} and iNPCs^{SU} were derived from the same individual, the iNPCs^{SU} were more similar to fNPCs derived from a different individual along with their respective differentiate cell types. The gene expression profiles depicted were performed with independent biological samples in duplicate.

Figure 6. Survival and integration of transplanted human iNPCs^{SU} in the rodent spinal cord (A–B) *In vitro* immunostaining of (A) iNPCs^{SU} and (B) fNPCs from identical batches of cells transplanted in rat spinal cords did not show FITC-conjugated Annexin V staining, nor pyknotic or condensed apoptotic nuclei (Hoechst), suggesting low levels of cell death. Rather the differentiated cells displayed robust astrocyte differentiation by GFAP staining. Scale bars: A, B = 50 μ m. (C–D) Representative confocal microscopy images of spinal cord histological sections from athymic nude rats (n=3–4 per group) transplanted with (C) iNPCs^{SU} and (D) fNPCs show adjacent co-staining of human cytoplasm (red, STEM123) and AQP4 (green). White cross-hairs show transplanted human cells that stained for both

AQP4 and human cytoplasm-specific STEM121 markers in the same focal plane. Adjacent panels show orthogonal views in yz and xz planes at the location of the white cross-hairs, confirming co-staining in three dimensions. Scale bars = 25 μm .

Figure 7. Engraftment of neural stem and progenitor cells in the rodent spinal cord (A–E) EZ spheres, (F–J) iNPCs^{AD}, (K–O) iNPCs^{SU}, and (P–T) fNPCs were transplanted into the spinal cord of athymic nude rats. Transplanted cells from each cell type survived to varying degrees and were observed at 7 and 21 days post-transplantation by STEM101, nestin and GFAP immunofluorescence staining in histological sections. Representative images with similar results are shown from $n=3-4$ animals that were transplanted in each group. (U) Graph showing cell concentration dosage (2×10^4 , 6×10^4 and 1×10^5) and cell survival of transplanted iNPCs^{SU} vs. iNPCs^{AD} as determined by stereological counting of

STEM101+ cells and 1/12th section sampling in 7 day post-transplantation spinal cords. Much greater cell survival was noted at all cell dosages in iNPCs^{SU}. *** = $p < 0.001$, ** = $p < 0.01$; * = $p < 0.05$; unpaired t-test (two-tailed). **(V)** Greater cell survival (STEM101+) was also observed in iNPCs^{SU} vs. iNPCs^{AD} at 21 day post-transplantation. However, no significant difference in survival was observed between fNPCs vs. iNPCs^{SU} or iNPCs^{AD} by stereological counting and 1/12th section sampling. * = $p < 0.05$; one-way ANOVA (Bonferroni post-test). **(U, V)** STEM101 counts: Coefficient of error (CE) range was 0.03–0.08 and markers counted were 193–2956. One sample in the 2×10^4 iNPCs^{AD} group had CE of 0.28 and 13 markers counted. **(W)** Proliferation of grafted cells (Ki67) was analyzed on tissue from animals having received cells at a concentration of 60,000 cells/ μ l. No significant differences were observed in cell proliferation by Ki67. CE ranges were 0.1–0.22 (Ki67) and 0.08–0.17 (STEM101). Markers counted 22–83 (Ki67) and 193–585 (STEM101). As expected, in all stereological counts in these data sets, the lowest CE and number of markers counted were observed in the iNPCs^{AD} samples associated with the very small grafts. **(X)** No significant differences were observed in cell migration between iNPCs^{AD}, iNPCs^{SU}, and fNPCs; one-way ANOVA. *** = $p < 0.001$, ** = $p < 0.01$; * = $p < 0.05$; unpaired t-test (two-tailed). Scale bars: **A, C, D, F, H, I, K, M, N, P, R, S** = 75 μ m and **B, E, G, J, L, O, Q, T** = 10 μ m. **(U–X)** n=3–4 rats per group were transplanted and analyzed.

Table 1

Antibody Characterization for Immunohisto/cytochemistry

Antigen	Immunogen / Band size on immunoblots	Dilution	Host/ Isotype	Company
A2B5	Embryonic Chicken Retinal Cells	1:500	Mouse IgM	Millipore
Aldh1L1	Full length protein(aa 1-902). Recognizes a band of 100 kDa.	1:25	Mouse IgG1	Antibodies, Inc.
Aquaporin 4 (AQP4)	CPDVEFKRRFKEAFSKAAQQTGKSYMEVDNRSQVETDDLKPGVVHVHIDVDRGEEKKGKQSGEVLSSV. Recognizes a band of 37 kDa.	1:2500	Rabbit	Sigma
GFAP	GFAP isolated from cow spinal cord. Recognizes a band of 50 kDa.	1:1000	Rabbit	Dako
GLAST (EAAT-1)	E. coli-derived recombinant human EAAT1/GLAST-1. His146-Ser237. Specific bands were detected at approximately 60 and 120 kDa for human neuroblastoma cell lines.	1:20	Sheep IgG	R&D Systems
Ki67	Synthetic peptide from C terminus of human Ki67. Recognizes a single band at 359 kDa.	1:500	Rabbit IgG	Abcam
NANOG	Synthetic peptide corresponding to residues near the amino terminus of human Nanog protein. Recognizes bands of a 42 kDa of 35kDa on iPS cell lysates.	1:400	Rabbit IgG monoclonal	Cell Signaling Technology
Nestin	Fusion protein. Recognizes 200-220 kDa band.	1:10,000	Rabbit IgG	Millipore
OCT3/4	Synthetic peptide (residues 300 to the C-terminus of human Oct4) conjugated to KLH.	1:250	Rabbit IgG polyclonal	Stemgent
OCT3/4-PE	Human Oct3/4 Isoform A Recombinant protein	1:100	Mouse IgG1, κ	BD Biosciences
S100B	Bovine brain S-100b. Recognizes an epitope located on the β chain (i.e. in S-100a and S-100b) but not on the α chain of S-100 (i.e. in S-100a and S-100a0). Predicted to recognize a 20 kDa band.	1:250	Mouse IgG1	Sigma
SOX2	Recombinant protein fragment containing a sequence corresponding to a region within amino acid 45 and 261 of human Sox2	1:100	Rabbit IgG polyclonal	Stemgent
SSEA4	Human embryonic carcinoma cell line 2102 Ep.	1:100	Mouse IgG3	Stemgent
SSEA4-AF647	Human Teratocarcinoma Cell Line.	1:100	Mouse IgG3	BD Biosciences
STEM101 (Ku80)	Human brain cell suspension.	1:200	Mouse IgG1	Stem Cell Inc.
STEM121	Recognizes human cytoplasm (see references in materials and methods).	1:2000	Mouse IgG1	Stem Cell Inc.
STEM123 (hGFAP)	Human peptide for sequence specific to human GFAP.	1:2000	Mouse IgG1	Stem Cell Inc.
TRA-1-60	Human embryonal carcinoma cell line 2102Ep	1:100	Mouse IgM	Stemgent
TRA-1-81	Human embryonal carcinoma cell line 2102Ep	1:100	Mouse IgM, κ	Stemgent
Tuj1 (βIII-tubulin)	Synthetic peptide corresponding to the C-terminal sequence of human β-tubulin isotype III coupled to BSA	1:1000	Mouse IgG2b	Sigma

Table 2

Primer sets qRT-PCR.

Genes	Accession	Sequences
<i>POU5F1 / OCT3/4</i> (CDS)	NM_002701.5 NM_203289.5	CCC CAG GGC CCC ATT TTG GTA CC
		ACC TCA GTT TGA ATG CAT GGG AGA GC
<i>POU5F1/OCT3/4</i> (pla)		CAT TCA AAC TGA GGT AAG GG
		TAG CGT AAA AGG AGC AAC ATA G
<i>SOX2</i> (CDS)	NM_003106.3	TTC ACA TGT CCC AGC ACT ACC AGA
		TCA CAT GTG TGA GAG GGG CAG TGT GC
<i>SOX2</i> (pla)		TTC ACA TGT CCC AGC ACT ACC AGA
		TTT GTT TGA CAG GAG CGA CAA T
<i>KLF4</i> (CDS)	NM_004235.4	ACC CAT CCT TCC TGC CCG ATC AGA
		TTG GTA ATG GAG CGG CGG GAC TTG
<i>KLF4</i> (pla)		CCA CCT CGC CTT ACA CAT GAA GA
		TAG CGT AAA AGG AGC AAC ATA G
<i>LIN28</i> (CDS)	NM_024674.4	AGC CAT ATG GTA GCC TCA TGT CCG C
		TCA ATT CTG TGC CTC CGG GAG CAG GGT AGG
<i>LIN28</i> (pla)		AGC CAT ATG GTA GCC TCA TGT CCG C
		TAG CGT AAA AGG AGC AAC ATA G
<i>L-MYC</i> (CDS)	NM_001033082.2 NM_001033081.2	GCG AAC CCA AGA CCC AGG CCT GCT CC
		CAG GGG GTC TGC TCG CAC CGT GAT G
<i>L-MYC</i> (pla)		GGC TGA GAA GAG GAT GGC TAC
		TTT GTT TGA CAG GAG CGA CAA T
<i>GAPDH</i>	NM_002046.4 NM_001256799.1	ACC ACA GTC CAT GCC ATC AC
		TCC ACC ACC CTG TTG CTG TA
<i>EBNA1</i> (pla)		ATC AGG GCC AAG ACA TAG AGA TG
		GCC AAT GCA ACT TGG ACG TT
<i>AQP4</i>	NM_001650.4 NM_004028.3	GCG AGG ACA GCT CCT ATG AT
		ACT GGT GCC AGC ATG AAT C
<i>GFAP</i>	NM_002055.4	CAC CAC GAT GTT CCT CTT GA
		GTG CAG ACC TTC TCC AAC CT
<i>GLAST/SLC1A3</i>	NM_001166695.1 NM_004172.4	ATC CTT GGA TTT ACC CTC CGA
		CGC CAT TCC TGT GAC AAG AC
<i>S100B</i>	NM_006272.2	TCC ACA ACC TCC TGC TCT TT
		GGA GAC AAG CAC AAG CTG AA
<i>SLC26A7</i>	NM_052832.3 NM_134266.1	AGA AGG CGA CTG CCC ATT TT
		ACT GCC AAC ATT ATC CCA GAC A
<i>SLC38A1</i>	NM_030674.3 NM_001077484.1	TGA CAG TGC CCG AGG ATG ATA
		TGG CTG TTT GTG AGA CTT CTT C

Genes	Accession	Sequences
<i>KROX20 (EGR2)</i>	NM_000399.3 NM_001136177.1	TGG CCG GAG ATG GCA TGA
		TAG GTG CAG AGA CGG GAG CA
<i>ISL1</i>	NM_002202.2	TCA CGA AGT CGT TCT TGC TG
		CAT GCT TTG TTA GGG ATG GG
<i>HOXB3</i>	NM_002146.4	CCA GTG CCA CTA GCA ACA G
		CGT TTG CCT CGA CTC TTT CAT C
<i>HOXB4</i>	NM_024015.4	ACG AGT CAG GGG TCG GAA TA
		CAT GGA GGG AAC TTG GGG TC
<i>SPRACL1</i>	NM_001128310.1 NM_004684.4	GCA CCT GAC AAC ACT GCA ATC
		TTT CAG CCT TAT GGT GGG AAT C
<i>NTRK2</i>	NM_006180.3 NM_001018064.1	TGT TCA GCA CAT CAA GCG ACA
		GCT CAG GAC AGA GGT TAT AGC AT
<i>FOXP1</i>	NM_005249.4	TGC GCA AAT GCC GCA TAA AT
		ACA CGG GCA TAT GAC CAC AG
<i>SIX3</i>	NM_005413.3	GTC CAT GGT ATT CCG CTC CC
		ATG GAG CGG TGG TGA GAA TC
<i>NKX2.1</i>	NM_001079668.2 NM_003317.3	GCC ATC TCC CGC TTC ATG
		CAG CGG GGC CAT GTT CTT G
<i>RPL13A</i>	NM_012423.3 NM_001270491.1	CCT GGA GGA GAA GAG GAA AGA GA
		TGA GGA CCT CTG TGT ATT TGT CAA

CDS; for detection of coding sequence gene expression.

Pla; for detection of plasmid vector-derived expression.