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Generation of GFAP::GFP astrocyte reporter lines from human adult fibroblast-derived iPS cells using zinc-finger nuclease technology

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Abstract

Astrocytes are instrumental to major brain functions, including metabolic support, extracellular ion regulation, the shaping of excitatory signaling events and maintenance of synaptic glutamate homeostasis. Astrocyte dysfunction contributes to numerous developmental, psychiatric and neurodegenerative disorders. The generation of adult human fibroblast-derived induced pluripotent stem cells (iPSCs) has provided novel opportunities to study mechanisms of astrocyte dysfunction in human-derived cells. To overcome the difficulties of cell type heterogeneity during the differentiation process from iPSCs to astroglial cells (iPS astrocytes), we generated homogenous populations of iPS astrocytes using zinc-finger nuclease (ZFN) technology. Enhanced green fluorescent protein (eGFP) driven by the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter was inserted into the safe harbor adeno-associated virus integration site 1 (AAVS1) locus in in disease and control-derived iPSCs. Astrocyte populations were enriched using Fluorescence Activated Cell Sorting (FACS) and after enrichment more than 99% of iPS astrocytes expressed mature astrocyte markers including GFAP, S100 β , NFIA and ALDH1L1. In addition, mature pure GFP-iPS astrocytes exhibited a well-described functional astrocytic activity in vitro characterized by neuron-dependent regulation of glutamate transporters to regulate extracellular glutamate concentrations. Engraftment of GFP-iPS astrocytes into rat spinal cord grey matter confirmed in vivo cell survival and continued astrocytic maturation. In conclusion, the

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Conflicts of interest statement

Nicholas J. Maragakis is an unpaid SAB member of Q Therapeutics Inc.; Pentti J. Tienari has a patent pending on “method for diagnosing neurodegenerative disease” based on the discovery of c9orf72; Jeffrey D. Rothstein and Rita Sattler have a patent filed on “composition for modulating C9orf72”.

generation of GFAP::GFP-iPS astrocytes provides a powerful in vitro and in vivo tool for studying astrocyte biology and astrocyte-driven disease pathogenesis and therapy.

Keywords

Induced pluripotent stem cells; GFAP; zinc finger nuclease; astrocyte; iPSC

Introduction

Astrocytes participate in a diverse range of CNS functions, including clearance/recycling of amino acid neurotransmitters, maintenance of K^+ homeostasis, energy metabolism, regulation of the blood-brain barrier and regional blood flow as well as a modulatory role in neuronal plasticity, neurogenesis and synapse formation (Allen and Barres 2009; Clarke and Barres 2013; Molofsky et al. 2012). Evidence for their participation in synaptic communication and network activity combined with their close anatomic opposition to pre- and post-synaptic elements has led to the model of the tripartite synapse (Araque et al. 1999). Consequently, proper function of this multi-cellular interplay is crucial for all neuronal activity along with suggested contributions to a large number of CNS disorders ranging from neurodegenerative diseases to psychiatric disorders (Ilieva et al. 2009; Molofsky et al. 2012).

In ALS, a uniformly fatal neurodegenerative disease characterized by progressive upper and lower motor neuron cell death, glial cells have long been known to contribute to disease pathogenesis in a non-cell autonomous manner (Boillee et al. 2006; Lobsiger et al. 2009; Philips and Rothstein 2014; Yamanaka et al. 2008a; Yamanaka et al. 2008b; Zhong et al. 2009). Oligodendroglia and astroglia have been investigated thoroughly in vitro and in vivo and play significant roles in ALS disease progression (Philips and Rothstein 2014). Astrocytes were first determined to be dysfunctional in CNS disease through loss of glutamate transporter EAAT2 leading to excitotoxic motor neuron cell death due to excessive accumulation of extracellular glutamate (Rothstein et al. 1992; Rothstein et al. 1990). Since then, extensive studies have addressed the detailed additional mechanisms of astrocyte contribution to disease pathogenesis in ALS. Diseased astrocytes obtained from rodent animal models (mice overexpressing human mutant superoxide dismutase, $SOD1^{mut}$ mice) as well as postmortem patient brain/spinal cord tissue are suggested to release toxic factors which in turn induce death of healthy, non-diseased motor neurons (Di Giorgio et al. 2007; Haidet-Phillips et al. 2011; Marchetto et al. 2008; Nagai et al. 2007). These findings were subsequently confirmed using co-cultures of human embryonic stem cell derived healthy motor neurons and $SOD1^{mut}$ mouse astrocytes (Di Giorgio et al. 2008) or human astrocytes expressing mutant $SOD1$ (Haidet-Phillips et al. 2011; Marchetto et al. 2008), supporting the significance of astrocyte dysfunction in ALS. Yet much of the work on disease pathogenesis is derived from mouse astroglia, while few studies are based on human cells with endogenous expression of the mutant gene(s).

There has been significant progress in human iPS cell generation using different strategies to reprogram human cells from different ages and origin since its discovery (Yamanaka 2007).

To date, numerous reports showed that neurological disease-specific iPSC cells have been successfully generated from patients' somatic cells (Hargus et al. 2014; Inoue et al. 2014; Peitz et al. 2013; Richard and Maragakis 2014), including ALS. The use of patient-derived iPSC cells to study disease pathogenesis represents a powerful novel research tool that may more accurately recapitulate the human genetic profile and allelic expression in disease. Several reports have described the differentiation of human astrocytes from iPSCs or embryonic stem cells (Emdad et al. 2012; Krencik et al. 2011; Mormone et al. 2014; Roybon et al. 2013; Sareen et al. 2014; Shaltouki et al. 2013). The commonly observed restrictions in using these differentiated astrocytes lies in the inherent cell culture heterogeneity of iPSC preparations. Not every iPSC cell undergoes equal differentiation and cell-type specific maturation, resulting often in cultures composed of heterogeneous cell types, including differentiated astrocytes, but also astrocyte progenitor cells and to a lesser degree, non-astrocyte cell types, e.g. neurons, microglia, oligodendroglia (Krencik and Zhang 2011; Roybon et al. 2013). This cellular heterogeneity can lead to a loss of important astrocyte signatures in heterogeneous culture preparations leading to functional, proteomic and transcriptomic level heterogeneities.

To overcome this problem, we generated GFAP::eGFP iPSC reporter lines (one healthy control and two ALS patient lines, SOD1^{A4V} and C9orf72) using zinc-finger nuclease-mediated insertion of an eGFP transgene in the AAVS1 locus of the *PPP1R12C* gene located on chromosome 19 (Sadelain et al. 2012). This site is considered a safe harbor locus and has been shown to lead to high levels of transgene expression (DeKolver et al. 2010; Hockemeyer et al. 2009; Papapetrou et al. 2011; Smith et al. 2008).

GFAP-driven expression of eGFP allows for enrichment of the differentiated eGFP-positive iPSC astrocytes using FACS. The purified iPSC astrocytes show astrocyte marker expression of GFAP and they are functionally mature as shown by the ability to respond to neuronal regulation of glutamate transport activity. Moreover, after engraftment into the rat spinal cord, astrocytes retained expression of GFAP-driven GFP, allowing for the human astrocytes to be distinguished and to be used for *in vivo* studies. In conclusion, these genetically modified astrocyte reporter lines represent a tool for future mechanistic investigation of astrocyte-driven disease pathogenesis and potential pre-clinical drug development for ALS and any other neurodegenerative disease characterized by astrocytic dysfunction.

Materials and Methods

iPSC generation and culturing

Patient fibroblasts were collected at Johns Hopkins Hospital with patient's consent (IRB protocol: NA_00021979) or by Pentti Tienari, MD at the Helsinki University Central Hospital. iPSC lines were generated as described previously (Donnelly et al 2013) and maintained in mTeSR1 (StemCell Technology). Three lines were used for the GFP cloning strategy: iPSC-Ctrl; iPSC-SOD1^{A4V}; iPSC-C9orf72 (for patient demographics see supplemental table S1).

Targeting vector design and integration

Enhanced green fluorescent protein (eGFP) and SV40 polyA sequence was cloned from pEGFP-N2 plasmid with some modifications. The Not I site between eGFP and SV40 polyA sequence was nulled and subsequently flanked by ClaI and NotI. This was followed by the cloning and ligation of a 7.5 kb promoter region of the human GFAP gene with eGFP and SV40 polyA sequence using EcoRI and ClaI sites. This cassette was then inserted into the targeting vector pZDonor –AAVS1 puromycin using EcoRI and NotI sites, flanked by 500bp left arm and 500 bp right arm for homologous recombination. The targeting vector total length reached about 16kb.

The integration of the targeting vector into iPSCs was performed following the manufacturer's protocol of the CompoZr Targeted integration-AAVS1 kit (Sigma-Aldrich). Briefly, 5 million iPSCs were transduced with 30µg targeting vector and 5µl ZFN mRNA using Nucleofector technology (Amaxa). Cells were treated with 2–5µg/ml puromycin for 12 days. Individual positive clones were picked using sterile pipette tips and transferred to 96-well plates for further identification. Puromycin-resistant clones were confirmed by PCR and Southern blotting (see also Figure 1a–c).

Preparation of genomic DNA

Genomic DNA was extracted using a Genomic DNA miniprep kit (for PCR, Sigma, # G1N70-1KT) or in combination with a Phenol/Chloroform extraction method (for Southern Blot). Briefly, one million cells were added to 180µl lysis solution C and 20µl proteinase K (100mg/ml, Sigma # P230810MG) solution and incubated at 70°C for 10 minutes. The sample mixtures were then extracted by Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v, Sigma # P3803-100ML) for 2–3 times and precipitated using 1/10 volume of 3M NaAc (pH5.3) and 2 volumes of ethanol. Pellets were washed with 75% ethanol once and dried. DNA was then resuspended in elution solution. DNA concentration and purity was determined using a Nanodrop 1000 machine (Thermo Scientific) and ethidium bromide-stained DNA on agarose gels, respectively.

PCR analysis to confirm correct transgene insertion

20 ng genomic cell DNA from puromycin-resistant clones, 2X Denville master mixture (Taq-Pro Red™ Complete kit, Cat #: CB4065-5), primers (250nM final concentration, see Table S2) and pure water were used for the PCR reaction. PCR conditions were as follows: 95°C for 3 min followed by 35 cycles of 94°C, 30 sec; 58°C, 30 sec; 72°C 90 sec, ending with 72°C for 2 min.

Differentiation of astrocytes from genetically modified iPS cells

Astrocytes were differentiated from iPSCs using previously described methods with minor modifications (Li et al. 2015; Roybon et al. 2013). Briefly, feeder-free iPS cells were cultured in *mTeSR*TM1 medium (Stemcell Technologies) with 20 ng/ml of bFGF (Life Technologies) on low attachment plates for 5 days. The medium was changed to Neural-induction Medium (Stemcell Technologies) supplemented with 2% B27 (Invitrogen), 1% glutamine (Life Technologies) and 40 ng/ml of bFGF. Media was partially changed every

two days for about 10 days. Neurospheres were mechanically dissociated into a single cell suspension and plated onto poly-L-ornithine (Sigma, 20 µg/ml)-laminin coated (BD Biosciences, 10 µg/ml) flasks. Cells were cultured in Astrocyte medium (ScienCell) for 30–45 days (passaged as necessary).

Fluorescent activated cell sorting (FACS)

Differentiated cells derived from ZFN-iPSC were harvested at day 90–97. Cells were washed once with PBS before and after 0.05% Trypsin/EDTA treatment for cell detachment (Life technologies, USA). Cells were resuspended in cold astrocyte medium (ScienCell Research Laboratories, USA) to obtain a single cell suspension (approximately $0.5\text{--}2 \times 10^7$ cells per milliliter) for sorting. The cells were analyzed and sorted on a MoFlo cell sorter using Summit software (both from Beckman Coulter, USA). GFP-positive cells were identified using 100nm nozzle with sheath setting at 25PSI and by a ratio of FL1 (530/30) and FL2 (580/30) fluorescence channels. An acquisition rate of 3,000–5,000 events per second was used as optimized conditions for astrocyte cell sorting.

Mouse primary neuron cultures

Primary cortical neurons were isolated from E16 embryonic mice cortices as described previously (Li et al. 2010). After dissociation with papain, one million neurons were seeded per well on top of a confluent layer of FACS purified human iPS astrocytes grown in a 6 well tissue culture plate. Co-cultures were maintained in Neurobasal medium supplemented with 5% FBS and 2% B-27.

Immunohistochemistry

iPS astrocytes were fixed with 4% paraformaldehyde followed by a blocking step with 5% normal goat serum (Vector labs) in PBS. Cells were incubated with primary antibodies in blocking solution overnight at 4°C: rabbit polyclonal anti-GFAP (1:200, Dako); Mouse anti-GFAP(1:500, Millipore); Rabbit anti-GFP(1:200, Abcam), Rabbit anti-SSEA3(1:200, Santa Cruz), Mouse anti-ALDH1L1 (1:5, Antibodies Inc./NeuroMAB 73-140; clone N103/39), Rabbit anti-NFIA (1:500, Abcam), Rabbit anti-synapsin 1 (1:500, Millipore), Rabbit anti-NMDAR1 (1:1000, Abcam), Rabbit anti-S100β(1:500, Dako); Rabbit anti-EAAT2(1:500, lab made); Rabbit anti-Iba1 (1:200, Wako); Rabbit anti-olig2 (1:200, Millipore); Rabbit anti-MAP2(1:500, Millipore). After repeated washing steps in PBS, cells were incubated with secondary antibodies: goat anti-rabbit IgG, Alexa Fluo 555 or 488 (1:500, Invitrogen); goat anti-mouse Alexa Fluo 488 (1:500, Invitrogen). Cells were washed 3 times for 5 min with 1× PBS and then 1 time with water and mounted using ProLong Antifade Gold with DAPI.

Transplanted processed rat tissue was blocked for 1 hr in 10% goat serum with 0.1% triton-X then probed overnight with primary antibody at 4°C in 2% goat serum with 0.1% triton-X. Tissue sections were washed in TBS then incubated with secondary antibody (Alexa Fluor, Life Technologies at 1:1000) for 2 hrs at room temperature followed by further washing in TBS. Tissue sections were mounted with Prolong Gold with DAPI (Life Technologies). Primary antibodies used: Human-specific GFAP (Stem Cells, Inc #STEM123 1:500), Human-specific cytoplasm (Stem Cells, Inc #STEM121 1:500), Anti-GFP (Millipore,

AB16901 1:1000), ChAT (Millipore, AB144P 1:100), Human Nuclei (Millipore, MAB1281 1:100), and Ki67 (Thermo Scientific, RM-9106-S, 1:400). The nuclear dye, To-pro-3 (Life Technologies, 1:1000), was added to TBS washes for some sections. Images were acquired on either a Zeiss fluorescence microscope using a Photometric Sensys KAF-1400 CCD camera (Roper Scientific) or on a Zeiss 700 laser confocal microscope. To quantify the percentage of transplanted cells proliferating *in vivo*, the transplanted cells were identified by HuNA immunohistochemistry and double stained for Ki67. For each image, the total number of double positive (HuNA⁺Ki67⁺) cells was counted and divided by the total number of HuNA⁺ cells per image to calculate the percentage of cells proliferating. Three 40X images were acquired at the site of transplantation for analysis and 3–4 rats per group were analyzed.

Southern Blot

At 5–10°C overnight with Xba I and separated by 0.8% agarose (Lonza, # 50004) at 85V(4v/cm) for 6 hrs (TAE buffer). Digested DNAs were depurinated for 30 min (in 400ml 0.25M HCl, slow shaking) and then denatured for 30 min with 400l denature (0.6M NaCl) followed by 30 min of neutralization with neutralizing buffer () Hybond-N+ membrane (GE health care, # RPN303B) using capillary transfer method. The blot was crosslinked with UV before hybridization (DNA-side-down, 254nm wavelength, 2 min). A 720bp PCR product was labeled with P32-dCTP (PerkinElmer, # NEG013Z500UC) for 1–2 hrs (Random primer DNA labeling system, # 18187013, Invitrogen.), purified with Illustra NICK columns Sephadex G50 (GE healthcare, # 17-0855-01), denatured (100°C for 10 min), then placed on ice for 10 min. The blot was first prehybridized in hybridization buffer (1M NaCl, 1%SDS, 10%Dextran Sulphate) at 65°C for 2–3 hrs and then hybridized at 65°C overnight with labeled probes (see supplemental table S2) and denatured salmon sperm DNA (Ambion, #AM9680, 75µM in the final buffer). I was washed twice with 2×SSC for 5 min at room temperature, followed by 0.2×SSC/0.1%SDS washing at 60°C for 15–30 min. DNA fragments were detected by autoradiography (Amersham Hyperfilm MP, GE healthcare, # 28906845). Both probes used for C9orf72 repeat expansion or ZFN targeting were confirmed by sequencing before used as a Southern blot probe.

Western blot

Cultured iPS astrocytes were lysed with lysis buffer (20 mM Tri-HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X and 0.1% SDS). The protein concentrations of the supernatants were quantified with DC protein assay kit (BioRad, CA). 10 µg of total protein per lane were loaded on precasting 10% polyacrylamide gel (BioRad) for SDS-PAGE. After being transferred onto PVDF membrane, immunoblots were probed with primary antibodies: anti-GLT-1 (1:1000), and anti-Actin (1:5000, Sigma); and subsequently incubated with HRP conjugated secondary antibody (1:10,000, GE Healthcare). Immunoblots were detected with SuperSignal West Pico chemiluminescent substrates (Thermo Scientific) and visualized by film. Intensities of bands were determined by ImageJ software.

Glutamate uptake

Glutamate uptake in iPS astrocytes was measured using 0.5 μ M L-glutamate (cold:radioactive=99:1) and 0.3 μ Ci L-[3 H]glutamate per sample (PerkinElmer). Cells grown in 6 well tissue culture plates were washed and pre-incubated at RT for 10 min in Na⁺ buffer (5 mM Tris-HCl, pH 7.2, 10 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM K₂HPO₄, and 10 mM D-glucose). Glutamate uptake was measured for 5 min at 37 C in Na⁺ buffer followed by two washes with ice-cold Na⁺-free assay buffer (5 mM TrisHCl, pH 7.2, 10 mM HEPES, 140 mM Choline-Cl, 2.5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM K₂HPO₄, and 10 mM D-glucose). To measure the amount of glutamate taken up, cells were lysed with 0.1N NaOH and [3 H] was measured using a liquid scintillation counter. Radioactive counts were normalized to total protein levels measured using the Bradford method.

Transplantation

Rats—Sprague-Dawley rats (9–10 weeks old, Taconic) were dosed daily with cyclosporine (20 mg/kg, Novartis) by subcutaneous injection beginning 3 days prior to transplantation and continued until sacrifice (Haidet-Phillips et al. 2014; Lepore et al. 2008). The care and treatment of animals in all procedures was conducted in strict accordance with the guidelines set by the NIH Guide for the Care and Use of Laboratory Animals, the Guidelines for the Use of Animals in Neuroscience Research and the Johns Hopkins University IACUC, and measures were taken to minimize any potential pain or animal discomfort.

Transplants—Rats were anaesthetized and cervical laminectomy performed as previously described (Haidet-Phillips et al. 2014; Lepore et al. 2008). Every rat received bilateral injections into the ventral horn at the 5th cervical spinal level (C5) for a total of 2 injections. Each injection contained 150,000 cells in a total of 2 μ l delivered using a 10 μ l Hamilton syringe secured to a micromanipulator with microsyringe pump controller (1 μ l/minute rate) (Lepore et al. 2008). For each cell line, four rats received transplants.

Tissue Processing: Rats were sacrificed at 1 month post-transplantation. Animals were anesthetized with 4% chloral hydrate and perfused with 0.9% saline, followed by ice-cold 4% paraformaldehyde (Fisher). Spinal cords were removed from the animal, followed by cryoprotection in 30% sucrose (Fisher)/0.1 M phosphate buffer at 4°C for 3 days. The tissue was embedded in Tissue Freezing Media (Triangle Biomedical Sciences), fast frozen with dry ice, and stored at –80°C until processed. Spinal cord tissue blocks were cut in the transverse planes at 30 μ m thickness. Sections were collected and stored at –20°C until analyzed.

Results

Targeting design for GFAP-eGFP iPS cell lines

To generate homogenous populations of iPS astrocytes, we introduced a GFAP-eGFP cassette into human fibroblast-derived iPSCs using zinc-finger nuclease technology. Expression of the eGFP reporter gene via activation of the astrocytic GFAP promoter would

allow us to use fluorescent cell sorting during the differentiation of iPSCs to iPS astrocytes and thereby obtain pure GFP-iPS astrocyte cultures.

Figure 1A illustrates the design of the targeting vector containing the eGFP reporter gene driven by a 7.5 kb promoter region of the human astrocyte specific *GFAP* gene, which has been shown previously to be sufficient for GFAP expression in astrocytes (Lee et al. 2008). The targeting vector also includes a puromycin antibiotic resistance gene to allow for clonal selection after successful insertion into the AAVS1 locus.

Three patient fibroblast-derived iPSC cell lines were used for the genetic targeting: (1) healthy, non-ALS and non-FTD control line; (2) ALS SOD1^{A4V} patient line and (3) ALS C9orf72 patient line (supplemental table S1). The iPSC cells were transfected with the targeting vector and zinc-finger nuclease mRNA via electroporation and grown in puromycin-containing cell culture media. Up to 100 puromycin-resistant clones per iPSC line were isolated and examined by PCR to ascertain proper gene targeting into the AAVS1 site. Figure 1B shows an example of a PCR screen of 10 individual clones from each iPSC line, confirming a homozygous insertion rate of 30–40%. Two positive clones from each line were further analyzed by Southern Blot probing for WT and genetically modified DNA (Figure 1C). No off-target activity during the genetic insertion of our targeting vector using ZFN was observed. Clones CTL #7, SOD1 #6 and C9orf72 #3 were chosen for continuing astrocyte differentiation and functional characterization.

Microscopic examination of the three transgenic iPSC (ZFN-iPSC) lines showed no morphological differences compared to non-transgenic iPSCs (Figure 1D) and all ZFN-targeted lines maintained pluripotency as indicated by positive immunostaining with pluripotency marker stage-specific embryonic antigen 3 (SSEA3; Figure 1E).

Differentiation of Human ZFN-iPSCs to Astrocytes

After successful insertion of the eGFP-targeting vector, differentiations of ZFN-iPSCs into iPS astrocytes from each line were initiated. Differentiation efficacy was monitored by immunostaining at different time points (Day 30, 60 and 90) using varying cell type specific markers, including: astrocytic (GFAP); neuronal (MAP2); microglial (Iba1) and oligodendroglial (Olig2). Previous studies in our laboratory confirmed that there is no overall difference in the ability of non-transgenic iPSCs to be differentiated into astrocytes based on the disease state of the cells (Li et al. 2015). For the present differentiations of transgenic iPSCs, we observed that after 30 days of differentiation, the cells lacked expression of any of the tested cell type markers, in addition to a lack of eGFP expression (data not shown). After 60 days of differentiation, only a small percentage of cells exhibited GFAP and eGFP expression (data not shown), while ZNF-iPS astrocytes at 90 days of differentiation showed greatly increased immunostaining for GFAP (28–48%) and eGFP (27–48%; Figure 2A+B), and no staining for MAP2, Olig2 and Iba1 (data not shown). Using FACS analysis we were able to quantify and confirm the percent of eGFP-positive astrocytes derived from ZNF-iPSCs: CTL: 54.7%; SOD1: 32.7% and C9orf72: 29.3% (Figure 2C). These data highlight the cell type heterogeneity present in differentiated iPS cell cultures and emphasize the need for FACS sorting to obtain pure iPS astrocyte cultures.

FACS leads to homogenous populations of GFP-iPS astrocyte cultures

To obtain a homogenous population of GFP-labeled iPS astrocytes, ZNF-iPS astrocytes were isolated using FACS at 85–95 days of differentiation. After sorting, cells were immediately placed back into culture flasks and continued to differentiate. Figure 3A shows representative immunostaining of purified GFP-iPS astrocytes for GFAP, GFP and S100 β at 7 days after FACS sorting (see also Supplemental Figure S1 for high power magnification images). As expected, nearly 100% of cells are GFAP positive in all three GFP-iPS astrocytes lines. Similarly, S100 β staining was present in 69–76% of cells and did not differ between control and disease GFP-iPS astrocytes. Additional astrocytic marker protein immunostaining revealed uniform expression of **aldehyde dehydrogenase family 1 member L1** (ALDH1L1) and nuclear factor I/A (NFIA) (Supplemental Figures S2+S3), similar to what has been shown in a previous study using overexpression of TagRFP in iPS astrocyte driven by the GFAP promoter (Holmqvist et al. 2015). In addition, immunostaining for neuronal, microglial and oligodendroglial markers was negative for all three iPS lines after FACS purification (data not shown), further validating the use of transgenic GFP-iPS astrocytes to obtain pure astrocyte cultures.

Purified GFP-iPS astrocytes acquire astrocyte-relevant functions

To ensure that the genetic manipulation of the ZNF-iPSCs did not affect astrocyte maturation or function, we performed several experimental paradigms on purified GFP-iPS astrocytes. Firstly, we tested purified astrocytes for their ability to upregulate the astrocytic excitatory amino acid transporter 2 (EAAT2) in the presence of neurons. EAAT2 is expressed at very low levels in vitro unless co-cultured with neurons (Gegelashvili et al. 2001; Munir et al. 2000; Schlag et al. 1998; Swanson et al. 1997; Yang et al. 2010; Yang et al. 2009). We therefore seeded primary mouse embryonic cortical neurons on top of a monolayer of GFP-iPS astrocytes at day 97 of differentiation. Co-cultures were maintained for 7 days before the cells were lysed and EAAT2 levels were quantified using Western blot analyses. Figure 4A shows a representative western blot for EAAT2 of all three GFP-iPSC astrocyte lines with and without co-cultured neurons, showing an increase in multimeric EAAT2 protein levels in the presence of neurons. Quantitative analysis of the immunoblotting revealed statistical increase of EAAT2 in CTL (1.7 fold) and C9orf72 (3.1 fold) iPS astrocytes, while SOD1 iPS astrocytes showed a 1.3 fold increase that did not reach significance (Figure 4B, N=3 wells). To ensure that the neuron-induced increased levels of EAAT2 represented functional glutamate transporters, we further measured glutamate uptake activity in the neuron-iPS astrocyte co-cultures and confirmed significant increase in glutamate uptake for all three GFP-iPS astrocyte lines (Figure 4C). Finally, we confirmed that co-culturing GFP-iPS astrocytes (healthy control and patient-derived) supported neuronal maturation as shown by the expression of mature synaptic marker proteins, such as glutamate receptor subtype GluN1 and synaptic marker protein synapsin-1 (Supplemental Figure S4).

Next, we investigated the stability of the (GGGGCC) n repeat expansion in the C9orf72 ZNF-iPSCs and the differentiated GFP-iPS astrocytes. Using southern blot analysis to detect this expanded repeat as shown previously in non-transgenic C9orf72 iPSCs and iPS neurons (Donnelly et al. 2013), we were able to confirm that the repeat size (about 10kb,

equivalent to 1280 GGGGCC repeats) of the chosen C9orf72 iPSC line did not change with the ZFN targeting and the astrocyte differentiation (Figure 4D). A higher molecular weight band of 15kb size and equivalent to ca. 2100 GGGGCC repeats appeared during the differentiation from iPSC to astrocytes, which reflects repeat instability occurring during the process of astrocyte differentiation.

Finally, to evaluate stability of the cultured cells over time, we tested the effects of freeze-thaw cycles on GFP expression. FACS isolated GFP-iPS astrocytes were frozen at day 95–98 of differentiation, stored in liquid nitrogen for up to four weeks and then thawed and re-cultured until confluency was reached. FACS analysis of thawed GFP-iPS astrocytes confirmed full recovery of GFAP-GFP expressing iPS astrocytes with a yield of GFP-positive cells between 97% and 99% (Figure 4E).

FACS-purified iPS astrocytes are transplantable in rats

Given that the ZFN-iPSC-derived astrocytes stably express GFP, these cells are ideally suited for *in vivo* morphological study of human astrocytes after xenograft into a rodent central nervous system. Therefore, we evaluated whether the iPSC-derived astrocytes could engraft into the rat spinal cord and retain expression of GFAP-driven GFP *in vivo*. We have previously shown iPSC-derived astrocyte progenitors can be engrafted into the rodent spinal cord and express mature astrocyte-specific genes *in vivo* (Haidet-Phillips et al. 2014). FACS-purified astrocytes differentiated from the ZFN-Control and ZFN-C9orf72 iPSC lines were transplanted to the ventral horn of the spinal cord of wild-type rats. At one month post-transplantation, the rats were sacrificed and spinal cords analyzed for the presence of the GFAP-GFP-expressing astrocytes. For both the ZFN-Control and ZFN-C9orf72 lines, the human astrocytes engrafted into the spinal cord grey matter and expressed GFAP-GFP *in vivo*. The GFAP-GFP co-localized with a marker for human-specific cytoplasmic GFAP confirming the observed GFP was restricted to expression from the transplanted human cells (Figure 5A, B). The engrafted human astrocytes distributed throughout the ventral horn of the spinal cord and expressed human-specific GFAP detected by immunostaining (Figure 5C). At high magnification, the human-specific GFAP co-localized with GFAP-driven GFP, verifying that the GFAP-GFP expression cassette faithfully reports GFAP expression *in vivo* (Figure 5D). The cells can be found adjacent to large ventral motor neurons, although the physiological significance is not known (Figure 5E). Although a proportion of the cells are mitotic at the time of transplant, only ~2% of the transplanted astrocytes expressed the proliferation marker Ki67 *in vivo* (Supplemental Figure S5). No gross differences were noted in astrocyte engraftment between the ZFN-Control and ZFN-C9orf72 lines. Thus, our purified population of transplantable astrocytes represents an ideal xenograft model system for further study of patient-derived astrocytes using an *in vivo* paradigm.

Discussion

We have now generated GFAP-GFP astrocyte-reporter cell lines of control, ALS and ALS/dementia patient-derived iPS cells using a single copy insertion protocol via homologous recombination and zinc-finger nuclease technology. The transgenic GFP-iPS astrocytes showed normal differentiation patterns, expressed mature astrocyte marker proteins and

showed functional astrocyte activity in vitro and could engraft to the rodent spinal cord after transplantation. The use of these pure human iPS astrocyte reporter lines will enable novel research paradigms on the role of astrocytes in neurodegenerative disease pathogenesis and will also provide a reliable cell culture platform for drug discovery.

Conventional site-specific gene targeting by homologous recombination in human stem cells is usually not efficient. Sangamo BioSciences has developed ZFNs, which has been applied in a variety of cell lines and organisms (Hockemeyer et al. 2009). ZFN-mediated gene targeting only requires relative short targeting arms (500bp for each arm for AAVS1 site) and results in about 30% targeting efficiency. Our work showed similar targeting efficiency despite the use of a larger targeting vector (15kb).

Human stem cells can be difficult for targeting via traditional homologous recombination methods, including the low efficient insertion of engineered constructs, which requires lengthy selection and screening protocols. Three recently developed techniques have provided powerful methods for genetic modification application: Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR), each with unique advantages respectively. Our goal was to generate a site-specific insertion of a fluorescent reporter gene in combination with a cell-specific promoter. Efficient and reliable methods for targeting and a need for a safe insertion site were the most critical issues for our choice. We therefore selected ZFN technology for our targeting strategy, which provided a safe harbor locus at the AAVS1 site (Gaj et al. 2013).

The choice of the GFAP promoter fragment to isolate pure astrocytes was based on the well characterized expression patterns and regulation of GFAP in astrocytes obtained both in vitro and in vivo. Although GFAP might not be expressed, at high levels in all subtypes of astrocytes, astrocytic GFAP expression has been reported in most CNS regions at embryonic and adult stage, therefore targeting the majority of CNS astrocytes (Lee et al. 2008; Yeo et al. 2013). Lee and colleagues (Lee et al. 2008) further determined that a 7.5kb promoter fragment size of GFAP ensures astrocyte-specific expression of eGFP. The differentiation of ZFN-iPSCs to astrocytes with the current protocol showed expression of GFAP at 90 days of differentiation, and all GFAP-GFP FACS isolated iPS astrocytes developed into mature, functional astrocytes, confirming the appropriate selection of this promoter region to obtain homogenous astrocyte populations. With a purity of 97–99% GFP-positive cells, this human astrocyte culture system is superior to any currently reported iPS astrocyte culture protocols in regards to homogeneity (Emdad et al. 2012; Krencik et al. 2011; Mormone et al. 2014; Shaltouki et al. 2013) and could therefore provide a more accurate model to study human ALS disease pathogenesis mediated by astrocytes in vitro or in vivo.

However one current limitation of these technologies is the generation of well-defined regional specific astroglia. To date there is little reliable and validated identification of molecular subtypes of adult human or rodent astroglia, other than the gross gray vs white matter morphological subtypes. Recently several groups have been able to developmentally define spinal cord astroglia subtypes in rodents (Hochstim et al. 2008; Molofsky et al. 2014) and only one recent study supports the ability to generate ventral midbrain and ventral spinal

cord human iPS astrocytes (Holmqvist et al. 2015). Fortunately the methods described here can be employed to eventually obtain regionally specific astroglia knowledge. Furthermore, rodent spinal cord astrocytes expressing mutant SOD1 engrafted to the wild-type rat spinal cord initiate degeneration of healthy motor neurons in vivo (Papadeas et al. 2011). GFP-iPS astrocytes can also engraft into the ventral horn grey matter, allowing for in vivo analysis of ALS patient-derived astrocytes and their interactions with motor neurons as well as future analysis of pathophysiology after transplantation of diseased iPS astrocytes into healthy rodents or therapeutic potentials after transplantation of healthy iPS astrocytes into diseased rodents.

In conclusion, the generation of these control and disease ZFN-iPSCs provides a set of novel tools that will allow for the study of ALS disease mechanisms in human cell cultures using a pure, homogenous cell population. The system can further be applied to any study examining the role of astrocytes in other diseases or physiological processes. Finally, the preparation of homogenous astrocyte cultures could provide a potential cell culture platform for astrocyte-specific drug discovery screening efforts, including those directed towards a personalized medicine therapeutic development approach by applying this technology to other patient-derived iPSC lines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Allen NJ, Barres BA. Neuroscience: Glia - more than just brain glue. *Nature*. 2009; 457(7230):675–677. [PubMed: 19194443]
- Araque A, Parpura V, Sanzgiri RP, Haydon PG. Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci*. 1999; 22(5):208–215. [PubMed: 10322493]
- Boillee S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron*. 2006; 52(1):39–59. [PubMed: 17015226]
- Clarke LE, Barres BA. Emerging roles of astrocytes in neural circuit development. *Nat Rev Neurosci*. 2013; 14(5):311–321. [PubMed: 23595014]
- DeKaveler RC, Choi VM, Moehle EA, Paschon DE, Hockemeyer D, Meijnsing SH, Sancak Y, Cui X, Steine EJ, Miller JC, et al. Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res*. 2010; 20(8):1133–1142. [PubMed: 20508142]

- Di Giorgio FP, Boulting GL, Bobrowicz S, Eggan KC. Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. *Cell Stem Cell*. 2008; 3(6):637–648. [PubMed: 19041780]
- Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci*. 2007; 10(5):608–614. [PubMed: 17435754]
- Donnelly CJ, Zhang PW, Pham JT, Heusler AR, Mistry NA, Vidensky S, Daley EL, Poth EM, Hoover B, Fines DM, et al. RNA Toxicity from the ALS/FTD C9ORF72 Expansion Is Mitigated by Antisense Intervention. *Neuron*. 2013; 80(2):415–428. [PubMed: 24139042]
- Emdad L, D'Souza SL, Kothari HP, Qadeer ZA, Germano IM. Efficient differentiation of human embryonic and induced pluripotent stem cells into functional astrocytes. *Stem Cells Dev*. 2012; 21(3):404–410. [PubMed: 21631388]
- Gaj T, Gersbach CA, Barbas CF 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013; 31(7):397–405. [PubMed: 23664777]
- Gegelashvili G, Robinson MB, Trotti D, Rauen T. Regulation of glutamate transporters in health and disease. *Prog Brain Res*. 2001; 132:267–286.
- Haidet-Phillips AM, Hester ME, Miranda CJ, Meyer K, Braun L, Frakes A, Song S, Likhite S, Murtha MJ, Foust KD, et al. Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. *Nat Biotechnol*. 2011; 29(9):824–828. [PubMed: 21832997]
- Haidet-Phillips AM, Roybon L, Gross SK, Tuteja A, Donnelly CJ, Richard JP, Ko M, Sherman A, Eggan K, Henderson CE, et al. Gene profiling of human induced pluripotent stem cell-derived astrocyte progenitors following spinal cord engraftment. *Stem Cells Transl Med*. 2014; 3(5):575–585. [PubMed: 24604284]
- Hargus G, Ehrlich M, Hallmann AL, Kuhlmann T. Human stem cell models of neurodegeneration: a novel approach to study mechanisms of disease development. *Acta Neuropathol*. 2014; 127(2):151–173. [PubMed: 24306942]
- Hochstim C, Deneen B, Lukaszewicz A, Zhou Q, Anderson DJ. Identification of positionally distinct astrocyte subtypes whose identities are specified by a homeodomain code. *Cell*. 2008; 133(3):510–522. [PubMed: 18455991]
- Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKolver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol*. 2009; 27(9):851–857. [PubMed: 19680244]
- Holmqvist S, Brouwer M, Djelloul M, Diaz AG, Devine MJ, Hammarberg A, Fog K, Kunath T, Roybon L. Generation of human pluripotent stem cell reporter lines for the isolation of and reporting on astrocytes generated from ventral midbrain and ventral spinal cord neural progenitors. *Stem Cell Res*. 2015; 15(1):203–220. [PubMed: 26100233]
- Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *J Cell Biol*. 2009; 187(6):761–772. [PubMed: 19951898]
- Inoue H, Nagata N, Kurokawa H, Yamanaka S. iPS cells: a game changer for future medicine. *EMBO J*. 2014; 33(5):409–417. [PubMed: 24500035]
- Krencik R, Weick JP, Liu Y, Zhang ZJ, Zhang SC. Specification of transplantable astroglial subtypes from human pluripotent stem cells. *Nat Biotechnol*. 2011; 29(6):528–534. [PubMed: 21602806]
- Krencik R, Zhang SC. Directed differentiation of functional astroglial subtypes from human pluripotent stem cells. *Nat Protoc*. 2011; 6(11):1710–1717. [PubMed: 22011653]
- Lee Y, Messing A, Su M, Brenner M. GFAP promoter elements required for region-specific and astrocyte-specific expression. *Glia*. 2008; 56(5):481–493. [PubMed: 18240313]
- Lepore AC, Rauck B, Dejea C, Pardo AC, Rao MS, Rothstein JD, Maragakis NJ. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci*. 2008; 11(11):1294–1301. [PubMed: 18931666]
- Li Y, Balasubramanian U, Cohen D, Zhang PW, Mosmiller E, Sattler R, Maragakis NJ, Rothstein JD. A comprehensive library of familial human amyotrophic lateral sclerosis induced pluripotent stem cells. *PLoS One*. 2015; 10(3):e0118266. [PubMed: 25760436]

- Li Y, Sattler R, Yang EJ, Nunes A, Ayukawa Y, Akhtar S, Ji G, Zhang PW, Rothstein JD. Harmine, a natural beta-carboline alkaloid, upregulates astroglial glutamate transporter expression. *Neuropharmacology*. 2010; 60(7–8):1168–1175. [PubMed: 21034752]
- Lobsiger CS, Boillee S, McAlonis-Downes M, Khan AM, Feltri ML, Yamanaka K, Cleveland DW. Schwann cells expressing dismutase active mutant SOD1 unexpectedly slow disease progression in ALS mice. *Proc Natl Acad Sci U S A*. 2009; 106(11):4465–4470. [PubMed: 19251638]
- Marchetto MC, Muotri AR, Mu Y, Smith AM, Cezar GG, Gage FH. Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell*. 2008; 3(6):649–657. [PubMed: 19041781]
- Molofsky AV, Kelley KW, Tsai HH, Redmond SA, Chang SM, Madireddy L, Chan JR, Baranzini SE, Ullian EM, Rowitch DH. Astrocyte-encoded positional cues maintain sensorimotor circuit integrity. *Nature*. 2014; 509(7499):189–194. [PubMed: 24776795]
- Molofsky AV, Krencik R, Ullian EM, Tsai HH, Deneen B, Richardson WD, Barres BA, Rowitch DH. Astrocytes and disease: a neurodevelopmental perspective. *Genes & development*. 2012; 26(9):891–907. [PubMed: 22549954]
- Mormone E, D'Sousa S, Alexeeva V, Bederson MM, Germano IM. Footprint-Free" Human Induced Pluripotent Stem Cell-Derived Astrocytes for In Vivo Cell-Based Therapy. *Stem Cells Dev*. 2014
- Munir M, Correale DM, Robinson MB. Substrate-induced up-regulation of Na(+)-dependent glutamate transport activity. *NeurochemInt*. 2000; 37(2–3):147–162.
- Nagai M, Re DB, Nagata T, Chalazonitis A, Jessell TM, Wichterle H, Przedborski S. Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci*. 2007; 10(5):615–622. [PubMed: 17435755]
- Papadeas ST, Kraig SE, O'Banion C, Lepore AC, Maragakis NJ. Astrocytes carrying the superoxide dismutase 1 (SOD1G93A) mutation induce wild-type motor neuron degeneration in vivo. *Proc Natl Acad Sci U S A*. 2011; 108(43):17803–17808. [PubMed: 21969586]
- Papapetrou EP, Lee G, Malani N, Setty M, Riviere I, Tirunagari LM, Kadota K, Roth SL, Giardina P, Viale A, et al. Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. *Nat Biotechnol*. 2011; 29(1):73–78. [PubMed: 21151124]
- Peitz M, Jungverdorben J, Brustle O. Disease-specific iPS cell models in neuroscience. *Curr Mol Med*. 2013; 13(5):832–841. [PubMed: 23642064]
- Philips T, Rothstein JD. Glial cells in amyotrophic lateral sclerosis. *Exp Neurol*. 2014
- Regan MR, Huang YH, Kim YS, Dykes-Hoberg MI, Jin L, Watkins AM, Bergles DE, Rothstein JD. Variations in promoter activity reveal a differential expression and physiology of glutamate transporters by glia in the developing and mature CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007; 27(25):6607–6619. [PubMed: 17581948]
- Richard JP, Maragakis NJ. Induced pluripotent stem cells from ALS patients for disease modeling. *Brain Res*. 2014
- Rothstein JD, Martin LJ, Kuncel RW. Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. *N Engl J Med*. 1992; 326(22):1464–1468. [PubMed: 1349424]
- Rothstein JD, Tsai G, Kuncel RW, Clawson L, Cornblath DR, Drachman DB, Pestronk A, Stauch BL, Coyle JT. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. *Ann Neurol*. 1990; 28(1):18–25. [PubMed: 2375630]
- Roybon L, Lamas NJ, Garcia-Diaz A, Yang EJ, Sattler R, Jackson-Lewis V, Kim YA, Kachel CA, Rothstein JD, Przedborski S, et al. Human Stem Cell-Derived Spinal Cord Astrocytes with Defined Mature or Reactive Phenotypes. *Cell reports*. 2013
- Sadelain M, Papapetrou EP, Bushman FD. Safe harbours for the integration of new DNA in the human genome. *Nat Rev Cancer*. 2012; 12(1):51–58. [PubMed: 22129804]
- Sareen D, Gowing G, Sahabian A, Staggenborg K, Paradis R, Avalos P, Latter J, Ornelas L, Garcia L, Svendsen CN. Human induced pluripotent stem cells are a novel source of neural progenitor cells (iNPCs) that migrate and integrate in the rodent spinal cord. *J Comp Neurol*. 2014; 522(12):2707–2728. [PubMed: 24610630]
- Schlag BD, Vondrasek JR, Munir M, Kalandadze A, Zelenia OA, Rothstein JD, Robinson MB. Regulation of the glial Na⁺-dependent glutamate transporters by cyclic AMP analogs and neurons. *Mol Pharmacol*. 1998; 53(3):355–369. [PubMed: 9495799]

- Shaltouki A, Peng J, Liu Q, Rao MS, Zeng X. Efficient generation of astrocytes from human pluripotent stem cells in defined conditions. *Stem Cells*. 2013; 31(5):941–952. [PubMed: 23341249]
- Smith JR, Maguire S, Davis LA, Alexander M, Yang F, Chandran S, French-Constant C, Pedersen RA. Robust, persistent transgene expression in human embryonic stem cells is achieved with AAVS1-targeted integration. *Stem Cells*. 2008; 26(2):496–504. [PubMed: 18024421]
- Swanson RA, Liu J, Miller JW, Rothstein JD, Farrell K, Stein BA, Longuemare MC. Neuronal regulation of glutamate transporter subtype expression in astrocytes. *J Neurosci*. 1997; 17(3):932–940. [PubMed: 8994048]
- Yamanaka K, Boillee S, Roberts EA, Garcia ML, McAlonis-Downes M, Mikse OR, Cleveland DW, Goldstein LS. Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice. *Proc Natl Acad Sci U S A*. 2008a; 105(21):7594–7599. [PubMed: 18492803]
- Yamanaka K, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, Misawa H, Cleveland DW. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci*. 2008b; 11(3):251–253. [PubMed: 18246065]
- Yamanaka S. Strategies and new developments in the generation of patient-specific pluripotent stem cells. *Cell Stem Cell*. 2007; 1(1):39–49. [PubMed: 18371333]
- Yang Y, Gozen O, Vidensky S, Robinson MB, Rothstein JD. Epigenetic regulation of neuron-dependent induction of astroglial synaptic protein GLT1. *Glia*. 2010; 58(3):277–286. [PubMed: 19672971]
- Yang Y, Gozen O, Watkins A, Lorenzini I, Lepore A, Gao Y, Vidensky S, Brennan J, Poulsen D, Won Park J, et al. Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. *Neuron*. 2009; 61(6):880–894. [PubMed: 19323997]
- Yeo S, Bandyopadhyay S, Messing A, Brenner M. Transgenic analysis of GFAP promoter elements. *Glia*. 2013; 61(9):1488–1499. [PubMed: 23832770]
- Zhong Z, Ilieva H, Hallagan L, Bell R, Singh I, Paquette N, Thiagarajan M, Deane R, Fernandez JA, Lane S, et al. Activated protein C therapy slows ALS-like disease in mice by transcriptionally inhibiting SOD1 in motor neurons and microglia cells. *J Clin Invest*. 2009; 119(11):3437–3449. [PubMed: 19841542]

Figure 1. Generation of GFAP-GFP iPSC reporter lines

(A) Schematic representation of the targeting vector. The relevant portion of the GFAP::eGFP was inserted into the intron 1 locus of *ppp1R12C* gene. Locations of primers for clone identification and of Southern blot probe are indicated. (B) iPSC clones carrying the correct insertion were identified by PCR. (C) Confirmation of targeted clones using Southern blot analysis. (D) Morphological examination of targeted iPSC clones using phase contrast microscopy. (E) Validation of ZFN-iPSCs using pluripotency marker SSEA3.

Figure 2. Differentiation of ZFN-iPSCs into GFAP-positive astrocytes

(A,B) Only a proportion of iPS astrocytes express GFAP or GFP at 90 days of differentiation. The non-GFAP/GFP positive cells do not stain positive for MAP2, Olig2 or Iba1 (M-O-I). (C) On day 90 of differentiation, between 30 and 54% of ZFN-targeted GFP-iPS astrocytes show expression of GFP and GFAP as shown by FACS analysis. Scale bars: 50 μ m; all images were taken at the same magnification.

Figure 3. Homogenous GFP-iPS astrocyte populations after FACS isolation

(A) Immunostaining of FACS purified astrocytes with astrocyte markers S100 β , GFAP and reporter gene GFP. (B) Quantification of the FACS isolated immunostained GFP-iPS astrocytes reveals 99% GFAP purity and between 69 and 76% S100 β positive cells. Scale bars, 50 μ m.

Figure 4. Differentiation of GFP-iPSCs into mature and functional astrocytes

(A) Western blot of astrocytic glutamate transporter EAAT2 in GFP-iPS astrocytes only and GFP-iPS astrocytes co-cultured with mouse neurons. EAAT2 is transcriptionally activated by neurons as shown by the increased protein expression levels in the co-cultures. (B) Quantification of the neuron-induced increase in astrocytic EAAT2 protein levels in GFP-iPS astrocytes. (C) Na⁺-dependent glutamate uptake was measured in GFP-iPS astrocytes alone and in the presence of primary mouse neurons. Similarly to the increase in EAAT2 protein levels, functional glutamate uptake is significantly increased in the presence of

neurons. * $p < 0.05$; ** $p < 0.01$ **(D)** Southern blot analysis of C9orf72 iPSCs and ZFN-iPSCs, as well as differentiated C9orf72 GFP-iPS astrocytes. While control iPSCs (CTL) only show a GGGGCC repeat size of about 2.5kb (about 25 repeats), C9orf72-positive iPSCs and iPS astrocytes maintain the presence of an expanded GGGGCC repeat during the genetic targeting and differentiation (15kb, about 2100 repeats). **(E)** FACS analysis of freeze-thawed GFP-iPS astrocytes to confirm viability of differentiated cells and maintenance of GFP-insertion during freeze-thaw cycles.

Figure 5. Engraftment of ZFN-iPSC-derived astrocytes into the wild-type rat spinal cord ventral horn

(**A, B**) Engrafted astrocytes at the site of injection express GFP-GFAP *in vivo* which co-localizes with a human-specific cytoplasm marker (hCyto). Low magnification (**A**), High magnification (**B**). Scale bars, 10 μ m. (**C**) The engrafted astrocytes fill the ventral horn grey matter at one month post-transplantation and express human-specific GFAP (hGFAP). The white dotted lines outline the rat spinal cord section and the spinal cord grey matter. Blue staining denotes DAPI. (**D**) Co-localization of GFP-GFAP and hGFAP in a single engrafted

astrocyte *in vivo* at high magnification. Scale bar, 10 μ m. **(E)** Engrafted astrocytes localized by human-specific cytoplasm (hCyto) are spatially localized adjacent to choline acetyltransferase⁺ (ChAT) motor neurons in the spinal cord ventral horn. Scale bar, 10 μ m.