

Gene Profiling of Human Induced Pluripotent Stem Cell-Derived Astrocyte Progenitors Following Spinal Cord Engraftment

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ABSTRACT

The generation of human induced pluripotent stem cells (hiPSCs) represents an exciting advancement with promise for stem cell transplantation therapies as well as for neurological disease modeling. Based on the emerging roles for astrocytes in neurological disorders, we investigated whether hiPSC-derived astrocyte progenitors could be engrafted to the rodent spinal cord and how the characteristics of these cells changed between in vitro culture and after transplantation to the in vivo spinal cord environment. Our results show that human embryonic stem cell- and hiPSC-derived astrocyte progenitors survive long-term after spinal cord engraftment and differentiate to astrocytes in vivo with few cells from other lineages present. Gene profiling of the transplanted cells demonstrates the astrocyte progenitors continue to mature in vivo and upregulate a variety of astrocyte-specific genes. Given this mature astrocyte gene profile, this work highlights hiPSCs as a tool to investigate disease-related astrocyte biology using in vivo disease modeling with significant implications for human neurological diseases currently lacking animal models. *STEM CELLS TRANSLATIONAL MEDICINE* 2014;3:575–585

INTRODUCTION

Stem cell transplantation strategies hold considerable promise in the understanding and treatment of a variety of neurological disorders. The in vitro use of neural stem cells has already gained attraction as a platform for recreating the sequences of neural development, modeling disease phenotypes, and for potential use in drug screening against target-disease pathways. However, the correlation between the in vitro characteristics of these cells for those tasks and the in vivo characteristics of those same cells for therapeutic transplantation studies as well as more complex in vivo disease modeling has only had limited investigation.

Various stem cell sources have been evaluated for brain and/or spinal cord engraftment studies, including human embryonic stem cells (hESCs), mesenchymal stem cells, fetally derived neural stem cells, and more recently human induced pluripotent stem cells (hiPSCs) [1–4]. Human iPSCs were initially reprogrammed from adult human fibroblasts and have been shown to be capable of differentiating into neural-specific cell lineages [5]. Enthusiasm for the potential of hiPSCs in treating neurological disorders has also grown, especially for diseases involving damaged or diseased glia, including spinal cord injury, multiple sclerosis, and other demyelinating disorders [1, 6]. The discovery that hiPSC-derived

oligodendrocyte progenitors can extensively remyelinate and rescue a mouse model of congenital hypomyelination has generated promise for both adult demyelinating and degenerative disorders as well as infantile oligodendroglial disorders [7]. Indeed, the first clinical trial involving transplantation of hESC-derived oligodendrocyte progenitors into patients with spinal cord injury was approved in 2009, paving the way for future trials involving the use of hiPSCs. Since then, a second trial testing transplantation of hESC-derived retinal pigment epithelium for macular degeneration has reported no safety concerns involving the use of these cells [8].

In addition to their promise as a cellular source for transplantation therapies, hiPSCs can also be used as valuable tools in the modeling of human disease [9, 10]. The generation of hiPSCs from living patients may be especially useful for neurological diseases for which diseased patient tissue is inaccessible through biopsy. Human iPSCs have already been generated from patients with a large variety of neurological disorders, and after differentiation toward neural cell fates, these hiPSCs have excitingly displayed disease-related phenotypes in vitro for a number of diseases [9, 10].

Although the majority of hiPSC work has focused on the production of neurons or oligodendrocytes from hiPSCs, it has become evident that

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astrocytes also play critical roles in both the healthy nervous system as well as in various conditions, notably amyotrophic lateral sclerosis (ALS), Rett syndrome, and Huntington's disease [11, 12]. Specifically, astrocytes expressing disease-linked mutant genes have been shown to directly cause neurotoxicity in these diseases. Because astrocytes are either damaged or pathologically altered in these disorders, transplantation of healthy astrocytes may limit neurotoxicity and provide additional support to injured neurons. In addition to the therapeutic goals of stem cell transplantation, generation and transplantation of patient hiPSC-derived astrocytes may be useful for in vivo disease modeling. This is especially true for diseases such as sporadic ALS, in which a genetic component has not been identified as the etiology in the majority of cases. Therefore, disease modeling by transplantation of patient-derived hiPSCs into the spinal cord may currently be one of the best means to study cell-specific disease mechanisms in vivo.

The goal of our study was to develop a transplantation paradigm for the engraftment of hiPSC-derived astrocytes for disease-modeling purposes as well as to evaluate the potential of these cells as a source for therapeutic transplantation. Because transplantation of hiPSC-derived neural stem cells yields mainly neurons or a mixture of neural cell types [13–15], we aimed to first differentiate hiPSCs into astrocyte progenitors in vitro and then evaluate their capacity to survive and engraft when transplanted into the ventral horn of the adult rodent spinal cord. In addition, a critical question to the stem cell transplantation field is whether progenitor cells continue to differentiate after transplantation and adopt mature properties in vivo, which may be essential for their potential therapeutic benefits in patients. In evaluation of the hiPSC-derived astrocyte progenitors, we used a novel human specific gene profiling approach to compare the in vivo expression profile between various stem cell lines after transplantation to the spinal cord. We also used this gene-profiling approach to compare the expression profiles of the astrocyte progenitors in vitro versus the expression profile after engraftment to evaluate how these cells mature in an in vivo environment.

Our data show that both hESC- and hiPSC-derived astrocyte progenitors are capable of engrafting and surviving in the rat spinal cord for at least 12 weeks. Immunohistochemical analysis and gene profiling of the transplanted human cells revealed the transplanted cells retain their astrocyte lineage in vivo with few cells from other neural lineages present. Additionally, gene profiling of the transplanted human cells shows that the astrocyte progenitors mature dramatically in vivo after transplantation and upregulate a variety of both structural and functional astrocyte genes. This observed maturation highlights their potential for use for in vivo disease modeling using patient-derived hiPSCs to study diseases of the brain and spinal cord.

MATERIALS AND METHODS

hiPSC Generation and Characterization

Retroviruses expressing Sox2, Oct4, and Klf4 were used to reprogram fibroblasts to create the 11a and 18c iPSC lines. The characterization of these iPSC lines has been previously described [16]. Briefly, these iPSC lines were evaluated for expression of the pluripotency factors alkaline phosphatase, NANOG, OCT4, SSEA3, SSEA4, TRA-1-60, and TRA-1-81. All iPSC lines were also analyzed for the ability to form three germ layers in an in vitro assay as well as the ability to form teratomas after transplantation into

immune-compromised mice. Details describing differentiation of hiPSCs are provided in the supplemental online data.

Rats

Sprague-Dawley rats (9–10 weeks old; Taconic, Hudson, NY, <http://www.taconic.com>) were dosed daily with cyclosporine (20 mg/kg; Novartis International, Basel, Switzerland, <http://www.novartis.com>) by subcutaneous injection beginning 3 days before transplantation and continued until sacrifice. The care and treatment of animals in all procedures were conducted in strict accordance with the guidelines set by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, the *Guidelines for the Use of Animals in Neuroscience Research*, and the Johns Hopkins University Institutional Animal Care and Use Committee. Measures were taken to minimize any potential pain or discomfort for the animals.

Transplants

Rats were anesthetized and cervical laminectomy was performed, as previously described [17]. Each rat received bilateral injections into the ventral horn at the sixth cervical spinal level (C6) for a total of two 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) [17].

NanoString Analysis

NanoString (NanoString Technologies, Inc., Seattle, WA, <http://www.nanostring.com>) analyses were performed at 12 weeks post-transplantation. Rats were perfused with 0.9% saline, and spinal cord tissue was harvested and fast-frozen in liquid nitrogen. RNA was isolated using TRIzol, followed by DNase treatment and RNA cleanup using RNeasy columns (Qiagen, Hilden, Germany, <http://www.qiagen.com>). As a control, RNA was also isolated using identical methods from three control human cervical spinal cords. Probe sequence and target genes used are given in supplemental online Table 1. For the NanoString assay, 100 ng of RNA was loaded per sample. For analyses comparing gene expression of transplanted cells across the spinal cord (supplemental online Table 2) as well as analysis of human spinal cord (supplemental online Fig. 7), all samples were normalized using internal positive controls, and the raw total counts after normalization were graphed. For analyses comparing gene expression between cell lines in vitro as well as analyses comparing gene expression in vitro versus in vivo (supplemental online Fig. 8; supplemental online Tables 3, 4), all samples were normalized using internal positive controls and four human-specific housekeeping genes (*B2M*, *GAPDH*, *GUSB*, and *OAZ1*), and total counts after normalization were graphed. Before normalization, all raw values reading less than 10 total counts were eliminated from analysis because they were under the standard limit of detection.

Statistical Analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, <http://www.graphpad.com>). Data in all graphs are represented as the mean \pm SEM. For immunohistochemistry analysis, we analyzed at least three injection sites for in vivo analysis and at least three wells for in vitro cultures. For transplantation studies, the number of animals used is represented for each cell line in Table 1, and two independent transplant sites were analyzed for all rats.

Table 1. Stem cell lines used for rat spinal cord transplantation

Cell line	Cell type	Reference	Age	Sex	Weeks until sacrifice (n) ^a		
					2	7	12
H13	hESC	Cowan et al. [18]	0	M	2	2	3
11a	hiPSC	Boulting et al. [16]	36	M	2	3	3
18c	hiPSC	Boulting et al. [16]	48	F	2	3	3

^an = number of rats receiving transplant of various cell lines.

Abbreviations: hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell.

RESULTS

Directed Differentiation of hESCs and hiPSCs to Astrocyte Progenitors

To assess astrocyte engraftment potential from hiPSCs and hESCs, we used two hiPSC lines created from healthy individuals as well as one hESC line for in vitro astrocyte progenitor generation (Table 1). All hiPSC and hESC lines had a normal karyotype, expressed pluripotency markers, and were capable of differentiating to all three embryonic germ layers [16, 18]. Astrocytes were generated from hESCs and hiPSCs using a recently characterized protocol for astrocyte differentiation [19]. Briefly, hiPSCs and hESCs were neuralized with BMP4 and activin/TGF- β antagonists, followed by caudalization and ventralization using retinoic acid and sonic hedgehog, respectively (Fig. 1A). By day 11 of differentiation, 75%–80% of the cells were neural progenitors expressing Pax6 and Sox2, indicating efficient neuralization (supplemental online Fig. 1A, 1B). As previously described [19], this protocol generates a mixture of immature Tuj1⁺ (β -tubulin) neurons and neural progenitors at early stages of differentiation before culture in glial differentiation media (supplemental online Fig. 1C, 1D). At day 30 of differentiation, the cells were transferred into glial differentiation media, including supplementation with 1% FBS. Astrocyte progenitors as defined by CD44 staining [20] were usually noticed by days 50–60 of differentiation. In addition, we stained for CD184, a newly described marker expressed by neural progenitors and astrocyte progenitors [21]. Our cells expressed CD184 by day 29 of differentiation (supplemental online Fig. 1C, 1D) and expressed the astrocyte progenitor markers CD184, CD44, S100 β , and Nestin after 100 days of differentiation, as previously described for this protocol (Fig. 1B, 1C) [19]. At this time point, between 30% and 50% of cells also expressed glial fibrillary acidic protein (GFAP) depending on the cell line (Fig. 1B, 1C). The majority of cells still expressed Nestin, CD44, S100 β , and CD184 at the end of the differentiation process, indicating the culture was a mixture of astrocyte progenitor cells and immature GFAP⁺ astrocytes. No NG2⁺ or Olig2⁺ oligodendrocyte lineage cells were observed in the cultures, and rare (<1%) Tuj1⁺ neurons could be identified after 100 days of differentiation, as previously described (Fig. 1C) [19]. Between 25% and 60% of the cells expressed Ki67 after 100 days of differentiation, indicating a proportion of the cells was mitotic at the time of transplantation (Fig. 1B, 1C).

Transplantation of hESC- and hiPSC-Derived Astrocyte Progenitors to the Rat Spinal Cord

To evaluate the astrocyte progenitors' propensity for engraftment, the cells were transplanted bilaterally to the ventral horn of the cervical spinal cord of adult wild-type rats. Before the injection and for the remainder of the study, rats were given high-dose cyclosporine

to prevent immune rejection of the grafted human cells. Rats were sacrificed at 2, 7, or 12 weeks post-transplantation (Table 1). All rats were observed daily, and no behavioral abnormalities were noted for the entirety of the study. At 2 weeks post-transplantation, cells could be localized in the spinal cord by staining for human-specific nuclear antigen (HuNA), and most of the transplanted cells resided within 1 mm rostral-caudal from the transplantation site (supplemental online Fig. 2). Evaluation of the transplanted cells at 7 weeks (supplemental online Fig. 3) and 12 weeks (Fig. 2A–2D) post-transplantation revealed the HuNA⁺ cells could be localized in the spinal cord at these time points with limited (<1 mm) rostral-caudal migration from the transplantation site. Quantification of HuNA⁺ cells in the spinal cord at 2, 7, and 12 weeks post-transplantation showed that the transplanted cells survived for up to 12 weeks, although survival was limited (<5% surviving at 12 weeks post-transplantation) (Fig. 2E). One reason that the quantified survival may be low is the limited proliferation of the cells in vivo (supplemental online Fig. 4). We also evaluated whether the transplanted HuNA⁺ cells were expressing markers indicative of apoptosis in vivo such as cleaved caspase-3; however, we could not detect expression of these markers at even 2 weeks post-transplantation. The quantified cell survival did not dramatically change between 2 and 12 weeks post-transplantation, suggesting either that the majority of cells do not survive in the first 2 weeks post-transplant or that many never engraft at the site of transplantation and are lost at the time of surgery. The remainder of the cells are engrafted long-term. The majority of transplanted cells resided in the gray matter of the spinal cord (Fig. 2F). No large differences in the survival and migration were noted between the different lines of hESCs and hiPSCs after transplantation at any of the time points examined. Additionally, no teratoma formation was noted in any of the rats at any time point examined.

Cell Lineage Characterization of Transplanted hESC- and hiPSC-Derived Astrocyte Progenitors

To determine whether the transplanted cells retained their astrocyte lineage after engraftment, we colocalized HuNA⁺ cells with various cell lineage markers to assess whether the human cells differentiated into GFAP⁺ astrocytes, Tuj1⁺ neurons, or Olig2⁺ cells of the oligodendrocyte lineage. At the time of transplantation, 30%–50% of the cells expressed GFAP depending on the cell line (Fig. 1C). However, after transplantation of the hiPSC lines and the hESC line, more than 80% of the transplanted HuNA⁺ cells expressed GFAP at 2 weeks post-transplantation (Fig. 3A). Immunohistochemical analysis of the transplanted cells at 7 weeks (supplemental online Fig. 5A) and 12 weeks (Fig. 3B, 3C) post-transplantation showed that more than 90% of the HuNA⁺ cells express GFAP at these time points, suggesting the majority of transplanted astrocyte progenitors differentiate within 2 weeks to GFAP⁺ astrocytes and remain stable for at least 12 weeks. To

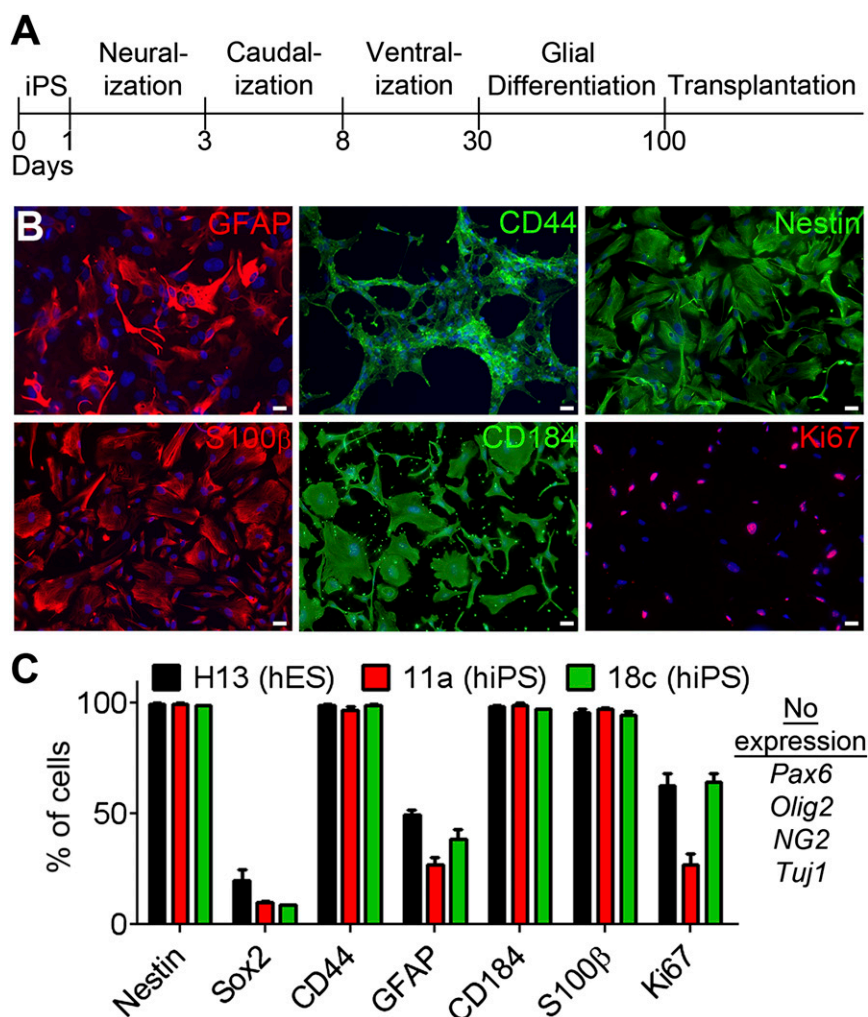


Figure 1. In vitro differentiation of human embryonic stem cells and human induced pluripotent stem cells into astrocyte progenitors. **(A):** Timeline for differentiation into astrocyte progenitors before transplantation. **(B):** Representative images of the hiPSC-derived astrocyte progenitors at the time of transplantation showing expression of astrocyte (GFAP), astrocyte progenitor (CD44, Nestin, S100β, CD184), and proliferation (Ki67) markers. Scale bars = 10 μm. **(C):** At the time of transplantation, immunocytochemical analysis shows all cell lines express markers indicative of an astrocyte progenitor phenotype. $n = 3$ wells per cell line analyzed. Error bars represent SEM. Abbreviations: GFAP, glial fibrillary acidic protein; hES, human embryonic stem cells; hiPS, human induced pluripotent stem cells; iPS, induced pluripotent stem cells.

verify the localization of the nuclear HuNA antigen with cytoplasmic markers such as GFAP, we also used an antibody specific for human mitochondria, which colocalized with GFAP (Fig. 3D). Additionally, the transplanted cells could be localized using a human-specific GFAP antibody that allowed us to observe their elaborate morphologies in vivo (Fig. 3E). The HuNA⁺ transplanted cells also expressed the astrocyte water channel, aquaporin 4, consistent with a mature astrocyte profile of gene expression (Fig. 3F). Few HuNA⁺/Olig2⁺ cells were noted (Fig. 3G), and no HuNA⁺Tuj1⁺ cells were observed in these lines, although transplanted cells resided in close proximity to endogenous rat neurons (supplemental online Fig. 5B). Overall, the astrocyte progenitors derived from different hESC and hiPSC lines did not significantly differ in their differentiation profile post-transplantation. Because only a fraction of the cells from the hESC and hiPSC lines expressed GFAP at the time of transplantation, these data suggest that the GFAP[−]/CD44⁺ fraction of cells differentiated in vivo to GFAP⁺ astrocytes within 2 weeks of transplantation. Alternatively, the GFAP⁺ fraction of cells

could have survived better after engraftment compared with the GFAP[−] fraction of cells. Another possibility is that the increase in GFAP reactivity indicates transition to a reactive astrocyte phenotype after transplantation. Therefore, we evaluated the transplanted cells for expression of LCN2, a recently identified marker for reactive astrocytes [22–24]. Although we could detect LCN2 in the spinal cord ventral horn of human ALS patients in which reactive astrogliosis is robust, we observed no LCN2 expression from HuNA⁺ transplanted cells, suggesting the transplanted astrocyte progenitors did not differentiate to a reactive phenotype in vivo (supplemental online Fig. 6).

In Vivo Gene Profiling of Transplanted hESC- and hiPSC-Derived Astrocyte Progenitors

To more fully characterize the phenotype of the transplanted human cells, we sought to develop a gene-profiling system to evaluate in vivo human-specific gene expression because there exist

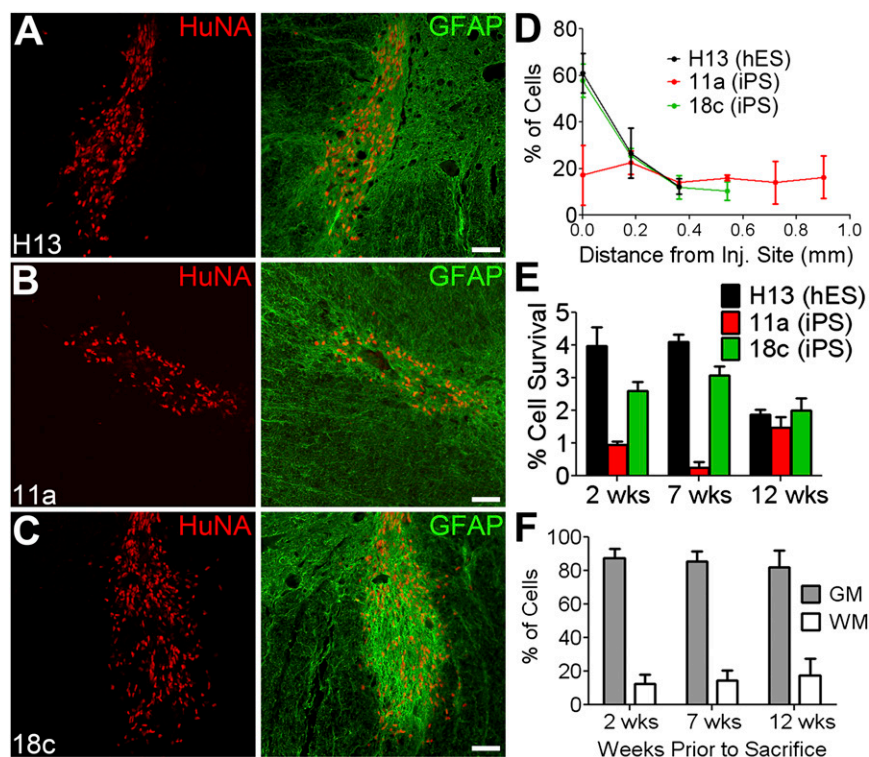


Figure 2. Characterization of human embryonic stem cell- and human induced pluripotent stem cell-derived astrocyte progenitors after transplantation to the rat spinal cord. **(A–C):** Human embryonic stem cell **(A)**- and induced pluripotent stem cell **(B, C)**-derived astrocyte progenitors can be localized by HuNA at 12 weeks post-transplantation, and many express GFAP in vivo. Scale bars = 50 μ m. **(D):** Rostral-caudal cell migration from the site of injection was measured at 12 weeks post-transplantation by calculating the percentage of total HuNA⁺ cells along the distance of the spinal cord. $n = 4$ injection sites analyzed per cell line. Error bars represent SEM. **(E):** Cell survival at 2, 7, and 12 weeks post-transplantation was quantified by counting the total number of HuNA⁺ cells surviving throughout the rostral-caudal extent of the spinal cord and dividing by the initial number of cells injected. $n = 4$ –6 injection sites analyzed per cell line. Error bars represent SEM. **(F):** GM/WM localization of transplanted HuNA⁺ cells was assessed at 2, 7, and 12 weeks post-transplantation by dividing the number of HuNA⁺ cells in GM or WM by the total number of surviving HuNA⁺ cells present throughout the rostral-caudal extent of the spinal cord. $n = 4$ –6 injection sites analyzed per cell line. Error bars represent SEM. Abbreviations: GFAP, glial fibrillary acidic protein; GM, gray matter; hES, human embryonic stem cells; HuNA, human-specific nuclear antigen; Inj., injection; iPS, induced pluripotent stem cells; WM, white matter.

limited human-specific antibodies appropriate for immunohistochemical analysis of astrocytes. For these studies, we used NanoString technology, which is a probe-based method for direct RNA quantification that allows for both sensitive and specific detection of small amounts of human-specific RNAs without cDNA conversion or further amplification steps [25]. We designed a custom panel of more than 50 genes (supplemental online Table 1), including well-described genes expressed by neural progenitor cells, neurons, oligodendrocytes, and astrocytes as well as less-characterized genes found to be expressed specifically in astrocytes in vivo by gene-profiling methods [26]. The design of human-specific RNA probes allows for measurement of gene expression levels specifically in the transplanted human cells within the rat spinal cord to create a gene expression profile for the engrafted human astrocyte progenitors.

For each line of transplanted hESC- or hiPSC-derived astrocyte progenitors, we analyzed in vivo gene expression at 12 weeks post-transplantation. We isolated RNA for gene expression profiling from the cervical spinal cord region at the injection site, from cervical sections rostral and caudal to the injection site, as well as from a lumbar section of the spinal cord, a remote site from the area of transplant. Because we observed few transplant-derived cells migrating even into the thoracic region of the spinal cord, we chose

the lumbar region as an area in which no human RNAs are present. The lumbar region was used to evaluate for any background cross-reactivity of the human-specific probes with endogenous rat RNAs.

Analysis of in vivo gene expression of transplanted astrocyte progenitors from the hiPSC and hESC lines showed that the transplanted cells expressed a wide array of astrocyte lineage genes in vivo (Fig. 4A–4C; supplemental online Table 2). These genes included structural astrocyte genes such as GFAP as well as functional genes expressed by mature in vivo astrocytes such as the water channel, aquaporin 4, the gap-junction protein, Connexin 43, the membrane-associated protein, MLC1, and the glutamate transporters, EAAT1 and to a lesser extent EAAT2 in some cell lines. For many of the astrocyte lineage genes, expression was highest at the transplant site and then decreased in a gradient fashion rostral and caudal from the transplant site (Fig. 4A–4C). We also evaluated human gene expression in three control postmortem human spinal cords and found a similar pattern of astrocyte gene expression, including high GFAP expression with lower but detectable expression of other astrocyte lineage genes such as aquaporin 4 and glutamate transporters (supplemental online Fig. 7).

Although we could detect high expression levels of a variety of astrocyte-specific genes, we did not detect any neuronal-specific genes (NeuroD, β -tubulin) expressed by the transplanted cells

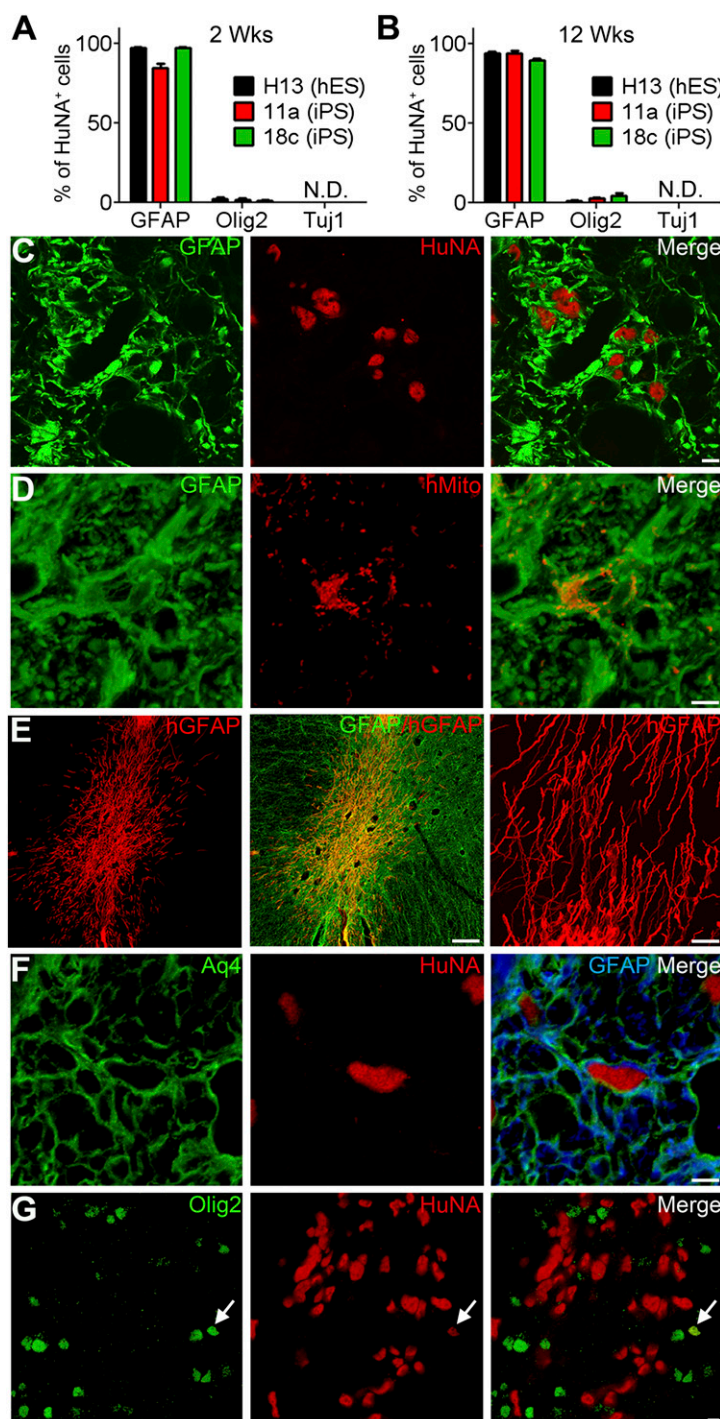


Figure 3. Transplanted human embryonic stem cell- and human induced pluripotent stem cell-derived astrocyte progenitors express astrocyte markers after transplantation to the rat spinal cord. **(A, B):** The percentage of HuNA⁺ cells that also express GFAP, Olig2, or Tuj1 was quantified by immunohistochemical analysis at 2 weeks **(A)** and 12 weeks **(B)** post-transplantation. $n = 4$ –6 injection sites analyzed per cell line. Error bars represent SEM. **(C):** Transplanted HuNA⁺ cells express GFAP and display typical astrocyte morphology at 12 weeks post-transplantation. Scale bar = 10 μ m. **(D):** Transplanted human cells show colocalization of hMito and GFAP at 12 weeks post-transplantation. Scale bar = 10 μ m. **(E):** The transplanted cells can be recognized using hGFAP and elaborate complex morphologies in vivo. Scale bar = 100 μ m. The far right panel shows hGFAP expression magnified at high power. Scale bar = 10 μ m. **(F):** HuNA⁺ cells colocalizing with GFAP also express the astrocytic water channel, Aq4. Scale bar = 10 μ m. **(G):** Few HuNA⁺ cells colocalize with the oligodendrocyte marker Olig2. Arrow denotes one HuNA⁺/Olig2⁺ cell. Scale bar = 10 μ m. Abbreviations: Aq4, aquaporin 4; GFAP, glial fibrillary acidic protein; hES, human embryonic stem cells; hGFAP, human-specific GFAP antibody; hMito, human mitochondrial marker; HuNA, human-specific nuclear antigen; iPS, induced pluripotent stem cells; N.D., not detected.

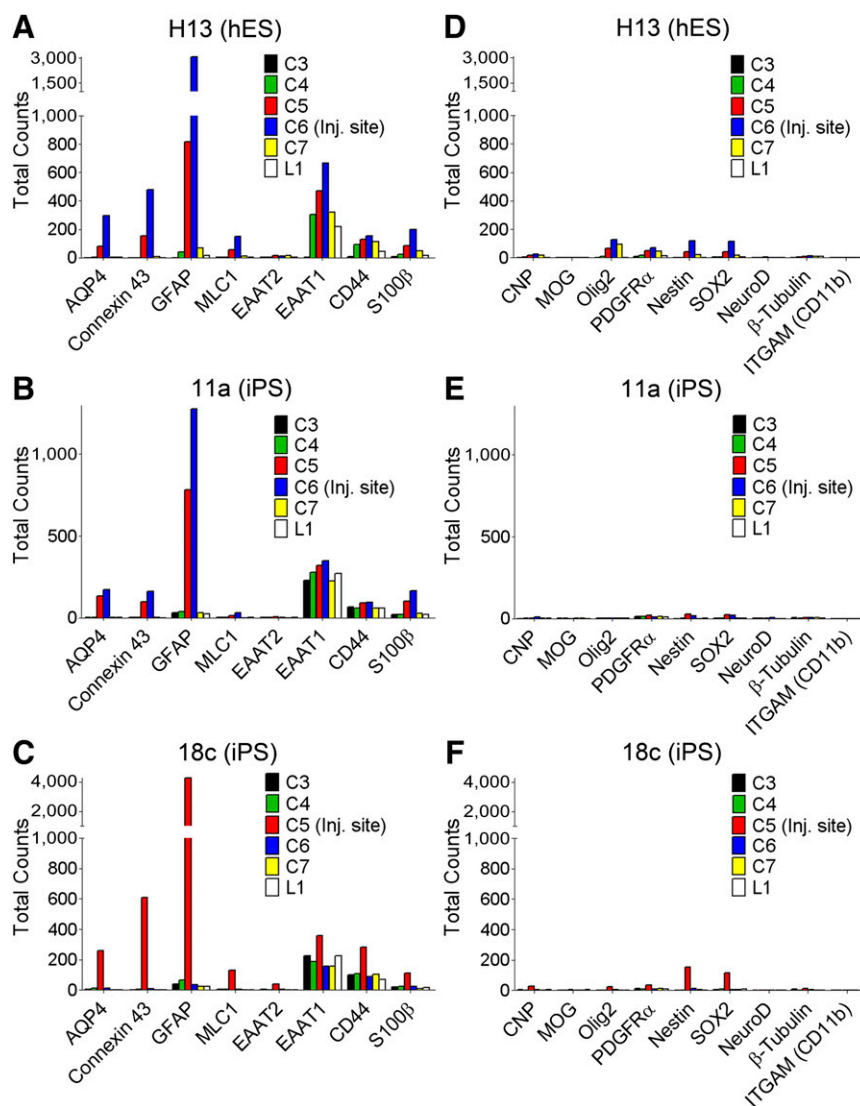


Figure 4. Human-specific *in vivo* gene profiling by NanoString analysis of transplanted human embryonic stem cell- and human induced pluripotent stem cell-derived astrocyte progenitors across cervical (C3–C7) and L1 lumbar segments of the spinal cord. (**A–C**): Gene profiling reveals expression of astrocyte lineage genes by transplanted H13 (**A**), 11a (**B**), and 18c (**C**) cells *in vivo*. RNA from separate spinal cord segments ranging from rostral (C3) to caudal (L1) from the site of injection was analyzed at 12 weeks post-transplantation. All samples were normalized using internal positive controls, and the raw total counts after normalization are graphed. (**D, E**): Gene profiling of nonastrocyte lineage genes expressed by transplanted H13 (**D**), 11a (**E**), and 18c (**F**) cells *in vivo*. RNA from separate spinal cord segments ranging from rostral (C3) to caudal (L1) from the site of injection was analyzed at 12 weeks post-transplantation. All samples were normalized using internal positive controls, and the raw total counts after normalization are graphed. Abbreviations: AQP4, aquaporin 4; EAAT1 excitatory amino acid transporter 1; EAAT2, excitatory amino acid transporter 2; GFAP, glial fibrillary acidic protein; hES, human embryonic stem cells; Inj., injection; iPS, induced pluripotent stem cells; MLC, megalencephalic leukoencephalopathy with subcortical cysts 1; MOG, myelin oligodendrocyte glycoprotein; NeuroD, neuronal differentiation 1; Olig2, oligodendrocyte lineage specific factor 2; PDGFR, platelet-derived growth factor receptor; SOX2, SRY (sex determining region Y)-box2.

(Fig. 4D–4F), consistent with the immunohistochemical results showing that the transplanted cells do not differentiate into neurons *in vivo* (Fig. 3B). Similarly, no transplant-derived expression of microglial-specific genes (ITGAM) was found (Fig. 4D–4F). However, we were able to detect human-specific transcripts for progenitor cell markers such as Nestin and Sox2, indicating some of the transplanted cells may reside in an immature progenitor state *in vivo* (Fig. 4D–4F). Likewise, we could also detect low levels of expression of early oligodendrocyte lineage genes such as Olig2, platelet-derived growth factor receptor- α , and CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase), which is consistent with the immunohistochemical analysis showing that a small percentage

(<5%) of cells differentiate toward the oligodendrocyte lineage *in vivo*. Mature oligodendrocyte genes related to myelin production such as myelin oligodendrocyte glycoprotein were not expressed by the transplanted cells at 12 weeks post-transplantation (Fig. 4D–4F). Overall, we did not notice large differences among the lines (H13, 11a, and 18c) in their *in vivo* gene profile after transplantation. The only noted difference was that the total level of astrocyte gene expression was lower in the 11a line compared with the 18c and H13 lines (Fig. 4A–4C). These results could indicate the 11a line did not mature as well into astrocytes as the other two cell lines. More likely though, these results reflect the slightly lower survival of this cell line after transplantation (Fig. 2E), with fewer cells

surviving corresponding to fewer human astrocyte transcripts detected overall in the spinal cord.

Gene Profiling Reveals Maturation of hESC- and hiPSC-Derived Astrocyte Progenitors Post-Transplantation

In addition to characterizing the *in vivo* gene expression profile of the transplanted cells, we furthermore asked how the gene expression profile of these cells compared with their expression profile *in vitro* just before transplant. For therapeutic transplantation purposes, it is crucial to know whether engrafted cells continue to differentiate *in vivo* and adopt the properties of mature astrocytes or whether they reside in an immature state after transplantation. Immunohistochemical analysis of the differentiated hESCs and hiPSCs *in vitro* showed that these cells express markers indicative of an astrocyte progenitor phenotype before transplant (GFAP, CD44, Nestin, S100 β , CD184) (Fig. 1C). *In vitro* gene profiling of the astrocyte progenitors before transplant also indicates these cells express both immature stem cell markers such as Nestin, Sox2, and CD44 as well as some astrocyte lineage genes such as GFAP, Connexin 43, and EAAT1 (supplemental online Fig. 8A, 8B; supplemental online Table 3). These gene-profiling results are consistent with the immunohistochemical data (Fig. 1C) indicating the highest level of GFAP expression in the H13 line, followed by the 18c line, and then the 11a line, with the lowest GFAP expression level before transplant (supplemental online Fig. 8A).

We sought to determine whether the expression of these astrocyte lineage genes increases after transplantation and whether the transplanted cells begin to express a more mature profile of astrocyte lineage genes after transplant. To compare the *in vitro* gene expression profile with the post-transplant gene expression profile, we normalized using human-specific housekeeping genes to specifically compare the level of human-specific gene expression *in vitro* versus *in vivo*. Using this method, we found that astrocyte-specific gene expression within the transplanted cells increased dramatically (note log scale on graphs) after engraftment to the rat spinal cord (Fig. 5; supplemental online Table 4). Structural genes such as GFAP were upregulated 100- to 500-fold (depending on the cell line) after transplantation, and some functional genes such as EAAT1 and EAAT2 were upregulated between 30- and 500-fold in most of the human cell lines *in vivo*. Of the top 20 astrocyte-specific genes found in a previous gene-profiling report of *in vivo* astrocytes [26], approximately half of these genes were increased after transplant in the 11a, 18c, and H13 lines (Fig. 5). Again, we noticed no striking differences in cell maturation between the different lines after transplant, although fewer astrocyte-specific genes were above the limit of detection in the 11a line (Fig. 5B).

In addition to examining whether astrocyte-specific genes increase after transplantation, we also analyzed the expression of a variety of genes that have been shown from mouse gene-profiling studies to increase or decrease as astrocytes mature from P7 to P17 *in vivo* [26]. We were interested to know whether our human stem cell-derived astrocyte progenitors expressed these genes before transplantation and whether they were upregulated after transplantation. In addition, we also analyzed expression of genes shown to be enriched in cultured mouse astrocytes compared with *in vivo* mouse astrocytes [26] to determine whether these genes are expressed in our cell lines *in vitro* and, if so, whether they are downregulated after transplantation.

In general, we saw that all of our hESC- and hiPSC-derived astrocyte progenitors before transplantation express a variety of

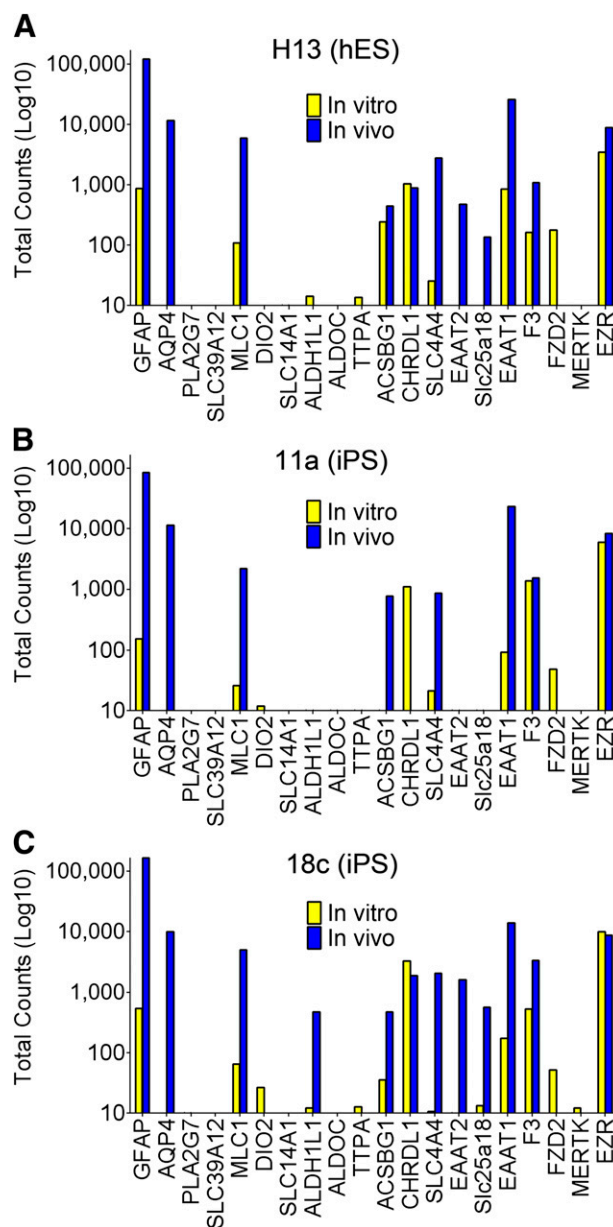


Figure 5. Human-specific gene profiling by NanoString analysis of human embryonic stem cell- and human induced pluripotent stem cell-derived astrocyte progenitors for astrocyte-specific genes *in vitro* before transplantation and *in vivo* after transplantation. **(A–C):** Gene profiling reveals increased expression of astrocyte-specific genes by H13 **(A)**, 11a **(B)**, and 18c **(C)** cell lines *in vivo* versus *in vitro*. RNA was isolated from each cell line just before transplant (*in vitro*) as well as from the *in vivo* spinal cord at the site of injection 12 weeks post-transplantation. All samples were normalized using internal positive controls and four human-specific housekeeping genes (*B2M*, *GAPDH*, *GUSB*, *OAZ1*). Total counts after normalization are graphed. The lack of a blue or yellow bar indicates that gene expression was below the limit of detection. Note: a logarithmic scale is used in all graphs of this figure. Abbreviations: AQP4, aquaporin 4; GFAP, glial fibrillary acidic protein; hES, human embryonic stem cells; iPS, induced pluripotent stem cells.

genes previously shown to be enriched in *in vitro* cultured astrocytes (Fig. 6). However, after transplantation, all of these genes, with the exception of *Anxa1*, were downregulated to undetectable levels within the human cells, indicating a phenotypic maturation post-transplantation (Fig. 6). Furthermore, a variety of

genes specifically enriched in developing astrocytes (P7) compared with mature astrocytes (P17) were also expressed in the hESC- and hiPSC-derived astrocyte progenitors before transplant (Fig. 6). Similar to the expression profile for in vitro culture-related genes, after transplantation, we observed a general downregulation of these genes in the human cells, and, in two of the transplanted cell lines (11a and 18c), we could not even detect many of these development-related genes in vivo (Fig. 6). In contrast, when we analyzed genes enriched in mature mouse astrocytes (P17) compared with P7 mouse astrocytes, we observed no clear pattern of upregulation or downregulation after transplant (Fig. 6). These observations may reflect differences in the development of mouse compared with human astrocytes in vivo as well as the observation that the mouse astrocyte genes were profiled from a forebrain population that may not be a general representation of all astrocytes.

DISCUSSION

One of the most significant advances in the stem cell biology field has been the generation of hiPSCs and development of protocols allowing differentiation of hiPSCs to specific cell lineages. Human iPSCs are promising for autologous cell transplantation therapies because they can be generated from a living patient and theoretically transplanted back into the same patient without concern of immune-mediated rejection based on genetic variations between the cells and the recipient. In addition, hiPSCs can be derived from patients with neurological disorders for disease-modeling purposes that may lead to further understanding of diseases for which genetic contributors are unknown or reliable mouse models are not available.

Based on the increasingly important role for glia in neurodegenerative disorders, we focused on hiPSC-derived astrocyte progenitors and transplantation of these cells to the spinal cord. Astrocytes expressing mutant disease-linked genes have been shown to directly contribute to neurotoxicity in ALS as well as in other diseases such as Rett syndrome [11, 12]. Thus, replacement of the aberrant astrocyte population in the spinal cord may be neuroprotective in ALS and other disorders. Additionally, for the majority of ALS patients, there is no known genetic contributor to the development of the disease (sporadic ALS). This presents difficulties in the study of basic disease-related mechanisms. In vivo disease modeling through engraftment of patient-derived hiPSCs to the rodent spinal cord may be one means to study astrocytes from these sporadic ALS patients in an in vivo environment. In vivo disease-modeling strategies have been attempted by transplantation of patient hiPSC-derived neurons for Huntington's disease [27], but similar measures using patient hiPSC-derived glia (rather than neurons) have not been attempted for ALS or other neurodegenerative diseases.

In this work, we investigated the propensity for engraftment of astrocyte progenitors derived from both hESC and hiPSC lines from healthy individuals as a first step toward characterizing these cells both in vitro and in vivo. Our data show that these astrocyte progenitors are capable of engrafting into the spinal cord and surviving long-term (at least 12 weeks) after transplantation. Although only a proportion of the cells expressed GFAP in vitro, post-transplantation, more than 90% of the cells expressed GFAP, indicating the astrocyte progenitors matured into astrocytes in vivo with very few cells of other lineages present. Further gene profiling at 12 weeks post-transplantation indicated the transplanted cells express a gene profile consistent with mature

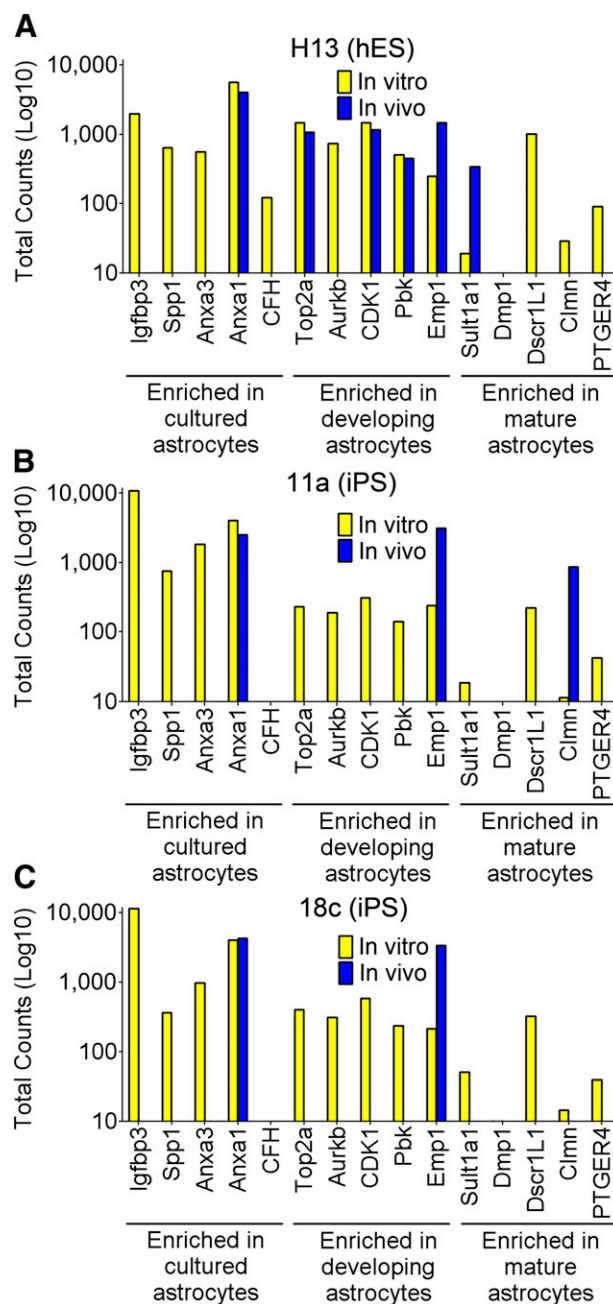


Figure 6. Human-specific gene profiling by NanoString analysis of human embryonic stem cell- and human induced pluripotent stem cell-derived astrocyte progenitors for genes involved in astrocyte development in vitro before transplantation and in vivo after transplantation. (A–C): Gene profiling reveals downregulation of many genes related to astrocyte in vitro culture and early development after transplantation of H13 (A), 11a (B), and 18c (C) astrocyte progenitors to the spinal cord. RNA was isolated from each cell line just before transplant (in vitro) as well as from the in vivo spinal cord at the site of injection 12 weeks post-transplantation. All samples were normalized using internal positive controls and four human-specific housekeeping genes (*B2M*, *GAPDH*, *GUSB*, *OAZ1*). Total counts after normalization are graphed. The lack of a blue or yellow bar indicates that gene expression was below the limit of detection. Note: a logarithmic scale is used in all graphs of this figure. Abbreviations: hES, human embryonic stem cells; iPS, induced pluripotent stem cells.

astrocytes *in vivo*, including upregulation of many astrocyte-specific genes after transplant.

One challenge we encountered in transplantation of the astrocyte progenitors was their limited survival and migration after transplant. Transplantation of less differentiated human fetal-derived and hiPSC-derived glial precursor cells results in much improved survival and migration, most likely a consequence of their proliferative capacity *in vivo* after transplant [7, 28, 29]. However, glial precursors have the ability to differentiate into astrocytes or oligodendrocytes, and this split differentiation profile was observed after transplantation of both fetal-derived and hiPSC-derived cells [7, 28, 29]. The resulting mixture of astrocytes and oligodendrocytes after transplant may present challenges in analyzing the specific contributions of astrocytes or oligodendrocytes in the context of *in vivo* disease modeling. In contrast, in the current study, we observed more than 90% of the hESC- and hiPSC-derived astrocyte progenitors differentiating into astrocytes *in vivo*. Therefore, transplantation of hiPSC-derived astrocyte progenitors may be more suitable for *in vivo* modeling of focal astrocyte-specific influences on spinal motor neurons in diseases such as ALS. Improvement in survival and migration of the astrocyte progenitors while retaining a restricted cell fate continues to present a challenge in using these cells for therapeutic purposes. Only one other study has described attempted transplantation of hESC-derived astrocyte progenitors; however, this study did not provide any quantitative data on the survival, migration, or differentiation profile of these cells *in vivo*, and these transplantation studies were also limited to hESC-derived astrocyte progenitors (not hiPSCs) [30].

As a result of the limited availability of human-specific antibodies, we tested a novel method to assess human-specific gene expression *in vivo* after transplantation using NanoString gene profiling. This gene-profiling approach allows for rapid assessment of *in vivo* gene expression using customizable human-specific probes, providing a snapshot of how the transplanted cells behave *in vivo* after transplantation. Using this method, we were able to show that the transplanted astrocytes express a wide array of astrocyte-specific genes *in vivo*, which would have been challenging by standard immunohistochemical measures.

Another goal of our work was to use gene profiling to assess how the transplanted cells change between *in vitro* culture versus after transplantation to the *in vivo* environment of the rodent spinal cord. Transcriptome analyses indicate a clear difference between astrocytes cultured *in vitro* versus *in vivo* endogenous astrocytes of the rodent forebrain [26]. Many of the perceived benefits of engrafted astrocytes rely on mature functions of these cells, which may or may not be present in the transplanted cells. For example, in ALS, astrocytes are well known to lose expression of the major glial glutamate transporter, EAAT2, causing excess glutamate to accumulate at the synapse and downstream neurotoxicity [31–33]. Our data show that the transplanted astrocyte progenitors express a variety of both structural (GFAP) and functional genes such as the water channel, aquaporin 4, the gap junction protein, Connexin 43, and glutamate transporters. Additionally, gene profiling demonstrates that the transplanted cells undergo significant maturation after engraftment, leading to upregulation of a variety of astrocyte-specific genes and downregulation of genes involved in astrocyte development. Because we only measured the gene profile of the transplanted cells out to 12 weeks post-transplantation, the possibility also exists that these cells will continue to mature and express even higher levels of

mature astrocyte genes at later time points *in vivo*. Still, these data highlight the potential usefulness for hiPSCs in *in vivo* modeling for diseases such as ALS in which astrocytes have been implicated in disease initiation and progression.

Lastly, although our understanding of hiPSCs has grown dramatically in recent years, much of this work has been limited to *in vitro* studies. Although Boulting et al. [16, 34–40] found considerable overlap in the neuronal differentiation propensity of hESCs and hiPSCs, several *in vitro* studies have suggested that hESCs differ significantly from hiPSCs and have also highlighted substantial variation between different lines of hiPSCs in gene expression and differentiation potential. We asked whether these variations exist only *in vitro* or whether cell line-specific differences are also present after transplantation to the spinal cord. In our work, all three cell lines that we transplanted behaved similarly *in vivo* with regard to survival, migration, and differentiation. We also noted no large differences between the hESC line and the two iPSC lines before or after transplant. Although our data include only three different cell lines, NanoString gene profiling may provide for a way to more quickly screen further cell lines to assess for line variability in survival, migration, and differentiation *in vivo*.

CONCLUSION

We have evaluated hiPSC- and hESC-derived astrocyte progenitors for their ability to engraft into the rodent spinal cord as the first step toward cell transplantation therapy and *in vivo* disease modeling using these cells. Although migration and initial survival were limited after transplantation of these cells, we did observe restricted differentiation to astrocytes *in vivo* with few cells from other lineages present. Our novel gene-profiling approach showed that hESC- and hiPSC-derived astrocyte progenitors continue to develop *in vivo* after transplantation and express mature astrocyte genes, suggesting the cells received *in vivo* environmental cues directing them toward terminal differentiation. Given the homogenous differentiation profile and mature gene expression of these cells, transplantation of patient-derived hiPSC astrocyte progenitors for *in vivo* disease modeling or for potential therapeutics in the spinal cord is feasible. Establishment of hiPSC-derived astrocyte progenitors as a tool for *in vivo* disease modeling may then lead to the discovery of novel mechanisms in ALS or other neurodegenerative disorders with astrocyte dysfunction.

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AUTHOR CONTRIBUTIONS

A.M.H.-P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; L.R.: conception and design, data analysis and interpretation, manuscript writing; S.K.G., A.T., J.-P.R., M.K., and A.S.: collection and/or assembly of data; C.J.D.: conception

and design, collection and/or assembly of data, data analysis and interpretation; K.E. and C.E.H.: provision of study materials, final approval of manuscript; N.J.M.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript, financial support.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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