

Generation of Neural Stem Cells from Embryonic Stem Cells Using the Default Mechanism: In Vitro and In Vivo Characterization

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Neural stem cell-based approaches to repair damaged white matter in the central nervous system have shown great promise; however, the optimal cell population to employ in these therapies remains undetermined. A default mechanism of neural induction may function during development, and in embryonic stem cells (ESCs) neural differentiation is elicited in the absence of any extrinsic signaling in minimal, serum-free culture conditions. The default mechanism can be used to derive clonal neurosphere-forming populations of neural stem cells that have been termed leukemia inhibitory factor-dependent primitive neural stem cells (pNSCs), which subsequently give rise to fibroblast growth factor 2-dependent definitive NSCs (dNSCs). Here we characterized the neural differentiation pattern of these two cell types in vitro and in vivo when transplanted into the dysmyelinated spinal cords of *shiverer* mice. We compared the differentiation pattern to that observed for neural stem/progenitor cells derived from the adult forebrain subependymal zone [adult neural precursor cells (aNPCs)]. dNSCs produced a differentiation pattern similar to that of aNPCs in vitro and in the *shiverer* model in vivo, where both cell types produced terminally differentiated oligodendrocytes that associated with host axons and expressed myelin basic protein. This is the first demonstration of the in vivo differentiation of NSCs, derived from ESCs through the default mechanism, into the oligodendrocyte lineage. We conclude that dNSCs derived through the default pathway of neural induction are a similar cell population to aNPCs and that the default mechanism is a promising approach to generate NSCs from pluripotent cell populations for use in cell therapy or other research applications.

Introduction

CELL-BASED THERAPIES USING neural stem cells (NSCs) to repair injured white matter in the central nervous system (CNS) have shown great promise as potential therapeutic approaches for models of traumatic CNS injury [1–4], neurodegenerative conditions [5,6], and congenital myelin disorders [7–9]. The successful translation of cell therapies relies not only on the efficacy of these approaches but also on the development of standardized, widely accepted techniques to derive cell populations (considering both the source and any methods of cell differentiation) that are of clinical grade. This will involve the rigorous control of reagents and the differentiation methods (and/or reprogramming methods) used to produce the cell populations, to maximize the safety profile of any given cell therapy.

Traditional techniques to derive NSCs and their derivatives from pluripotent cell populations involve the induction of embryoid body (EB) formation [10] by the withdrawal of growth factors promoting pluripotency, followed by the selection and subsequent enrichment of ectodermal and ultimately neural cells. The most common techniques have been based on EB formation followed by retinoic acid treatment [3,11]. Less common methods involve co-culture with stromal cell populations with neural inducing properties [12]. Despite the success of these approaches, the involvement of EB formation creates a risk for the presence of non-neural lineage cells in later cell populations. This can lead to tumorigenic potential, as has been observed in oligodendrocyte progenitor derived from murine embryonic stem cells (ESCs) by EB formation followed by treatment with retinoic acid, noggin, fibroblast growth factor (FGF2), and platelet-derived growth factor (PDGF) [13]. The maintenance of non-neural

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lineage cells in these cultures can likely be traced to the nonspecific differentiation pattern initiated in the process of cell aggregation leading to EB formation. To address this concern, we used the default pathway of neural induction [14–16] to produce clonal NSC populations directly from ESCs without EB formation.

Our method is based on an intrinsic property of ESCs, whereby the neural lineage is the default pathway of ESC differentiation in the absence of other extrinsic signaling (Fig. 1A) [14,15,17]. This default mechanism has been shown to act in vivo as well as functioning in vitro in ESCs (for review see [16]). The default model of neural induction is based on findings that neural lineage commitment is induced through the inhibition of bone morphogenetic protein signaling through both protein inhibitors and/or modified gene expression and, in the absence of any instructive signaling, ectodermal cells adopt a neural fate [16,18]. Recent findings suggest an important role for the zinc finger protein Zfp521 in this process [19]. When cultured in low density minimal conditions, ESCs rapidly acquire a default neural identity that is seemingly similar to adult neural stem or progenitor cells, as judged by the expression of the neural markers nestin and Sox1 by over 99% of surviving cells after 24 h [14]. Using a limiting dilution cell assay [15], similar to that used to generate neurosphere colonies of adult neural precursor cells (aNPCs) from the adult brain, it has been demonstrated that a small percentage (~0.2%) of ESCs possess the ability to generate NSC colonies when cultured at low density, in defined serum-free media (SFM), and in the presence of

leukemia inhibitory factor (LIF). This cell population was termed the primitive NSC (pNSC). While evidence for the adoption of a neural fate is strong when culture conditions are extremely minimal, when pNSCs are cultured to produce neurospheres in the presence of LIF, the commitment to the neural lineage is incomplete. Cells are found to still express the pluripotency marker octamer-binding transcription factor 4 (Oct4) and possess multi-germline potential in mouse embryo chimera experiments [15]. However, when the primary neurosphere colonies are passaged, they develop dependence on exogenous basic FGF2, similar to that observed in aNPCs; they no longer express Oct4; and they lose the ability to incorporate into the developing blastocyst in chimera experiments. These secondary neurosphere colonies have been named definitive NSCs (dNSCs) [14,15]. Here, we hypothesized that the generation of dNSCs through the default pathway would act as a useful method to generate NSCs from a pluripotent cell population with a therapeutic potential similar to that of aNPCs for cell-based remyelinating therapies. In this study we performed gene expression profiling of pNSC, dNSC, and aNPC neurosphere colonies, and characterized the neural differentiation pattern of NSC populations that were derived through the default pathway both in vitro and in vivo, after transplantation into the dysmyelinated spinal cords of *shiverer* mice. We compared the differentiation pattern of the pNSCs and dNSCs to that of aNPCs. The results suggest that the production of NSC from ESCs, through the default pathway, is a promising method to produce neural lineage cells for the develop-

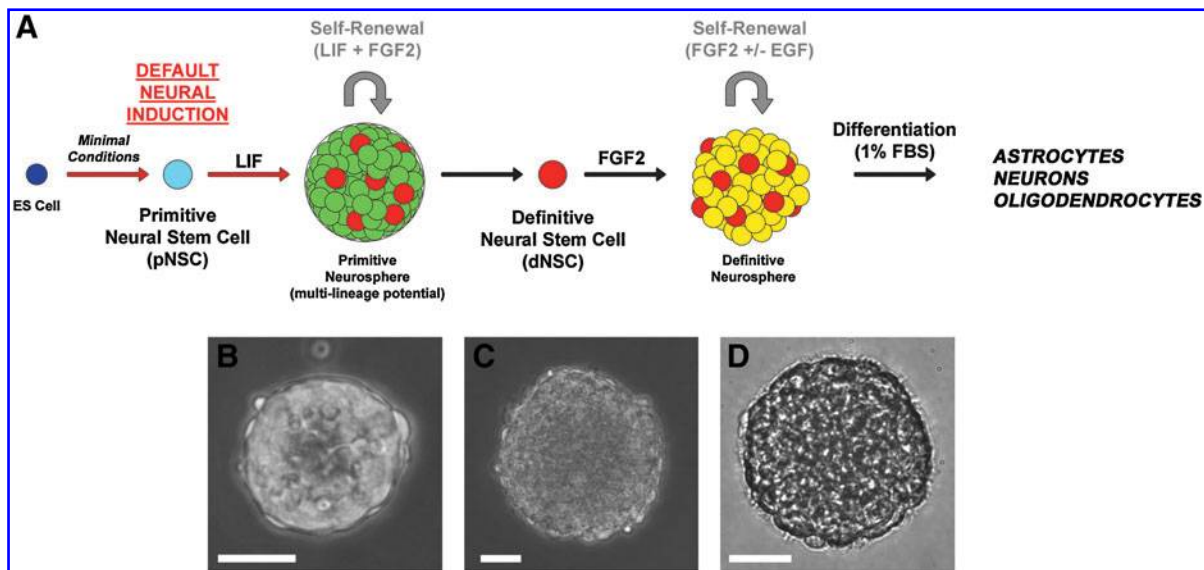


FIG. 1. Default neural differentiation of embryonic stem cells. **(A)** Schematic representation of neural induction of ESCs through the default pathway: Individual ESCs when cultured at low density in minimal serum-free media containing of LIF acquire a neural identity through a default mechanism. These neural stem cells colonies are termed pNSCs, are LIF-dependent, and divide to form clonally produced floating neurosphere colonies. pNSC-derived neurospheres can be dissociated into single cells and passaged indefinitely in serum-free media containing LIF or can be passaged into serum-free media containing FGF2 to produce a distinct population of FGF2-dependent cells termed dNSCs that also divide to form clonally produced neurospheres. These spheres can also be passaged indefinitely and when differentiated produce all three cell types of the neural lineage. **(B)** Phase-contrast image of a neurosphere colony generated by a pNSC. **(C)** Phase-contrast image of a neurosphere colony generated by a dNSC. **(D)** Phase-contrast image of a neurosphere generated by adult neural stem cells derived from the subependymal layer of the adult mouse forebrain. For **B–D**, bar equals 50 μ m. ESCs, embryonic stem cells; LIF, leukemia inhibitory factor; dNSC, definitive neural stem cell; pNSC, primitive neural stem cell; FGF2, basic fibroblast growth factor; Olig2, oligodendrocyte transcription factor 2.

ment of cell-based remyelinating therapies or other research purposes.

Materials and Methods

All experimental animal procedures were approved by the animal care committee of the University Health Network and are in accordance with the policies and guidelines of the Canadian Council on Animal Care.

ESC culture and production of ES-derived neurospheres through the default mechanism

All ES-derived neurospheres colonies were produced using previously reported techniques [14,15,20]. The ESC line used was the YC5 variant of the R1 mouse ESC line [15] that expresses yellow fluorescent protein (YFP). To produce primary neurospheres generated by pNSCs, ES colonies grown under standard ES conditions on feeder cells were dissociated using Trypsin–ethylenediaminetetra-acetic acid (Sigma) and seeded in uncoated plastic culture flasks into chemically defined SFM [21] containing LIF (1,000 U/mL) at a density of 10 cells/ μ L. At this cell density, the primary spheres produced have been previously demonstrated to be clonally derived [22]. Primary spheres were present after 7 days, passaged as described above, and plated at 10 cells/ μ L into SFM containing LIF (Millipore), FGF2 (10 μ g/100 mL; Sigma), and Heparin (2 μ g/100 mL; Sigma). Secondary spheres were present after 7 days and could be passaged to derive tertiary sphere populations. Definitive neurospheres, derived from dNSCs, were produced by the passage of single cell dissociated LIF-dependent pNSC spheres, at passage 3, into SFM containing FGF2 and Heparin, but not LIF. B27 nutrient supplement (Gibco) was added to enhance cell survival in the absence of LIF [14] (Fig. 1A).

Adult NSC isolation and culture

aNPCs were obtained from the brains of transgenic mice expressing enhanced YFP (EYFP) [129-Tg (ACTB-EYFP) 2Nagy/J; The Jackson Laboratory]. Cells were harvested from the forebrain subependymal zone using previously described methods [1,23,24]. Primary neurospheres were present after approximately 7 days after initial isolation and passaged weekly by mechanical dissociation (no trypsin) and were seeded into SFM containing FGF2, epidermal growth factor (EGF), and heparin at a density of 10–20 cells/ μ L.

In vitro cell differentiation and immunocytochemistry

For the in vitro analysis of cell fate potential, neurospheres were differentiated by removing growth factors (LIF, FGF2, Heparin, and EGF) from the media and adding 1% fetal bovine serum, as previously reported [1]. All differentiation experiments were conducted using neurospheres between passages 3 and 5. The spheres were dissociated as described and seeded onto Matrigel (Sigma)-coated, multi-well glass slides (Thermo Scientific/Nunc). Wells were seeded at 5,000 cells per well for pNSCs and 15,000 cells per well for dNSCs and aNPCs. Cells were allowed to differentiate for 7 days before being fixed for immunocytochemical analysis with 4% paraformaldehyde (PFA) at room temperature. Nonspecific binding of antibodies was blocked by incubation for 1 h with phosphate-buffered saline (PBS) containing 1% bovine serum

albumin (BSA), 10% normal goat serum (NGS), and 0.25% Triton X-100. Cultures were treated with primary antibodies (Table 1) for 2 h at room temperature and washed 3 times before incubation with appropriate fluorescently conjugated secondary antibodies for 1 h. Nuclei were counterstained by the use of VECTASHIELD mounting medium with 4',6'-diamidino-2-phenylindole (DAPI; Vector Labs).

Real-time PCR

Real-time reverse transcription–polymerase chain reaction (RT–PCR) analysis was conducted using TaqMan-based preloaded microfluidic array cards and the ABI PRISM[®] 7900HT Sequence Detection Systems (Applied Biosystems) to generate a limited gene expression profile for neurospheres produced by each NSC type. TaqMan cards were used according to the manufacturers' instructions and all PCR assays were performed in triplicate. RNA was isolated using Trizol for initial cell disruption, followed by the commercially available RNeasy Micro kit (Qiagen). RNA was isolated from intact sphere colonies at passage 3. Gene expression in the ESC line, used to produce the pNSC and dNSC spheres, was used at the baseline expression level for calculating relative expression values. The expression was normalized to glyceraldehyde 3-phosphate dehydrogenase. ES colonies used for RNA extraction were grown on matrigel-coated dishes in embryonic fibroblast-conditioned ES media, instead of on feeder cells. Gene expression levels were compared using the $2^{-\Delta\Delta CT}$ method [25] using the Applied Biosystems SDS software package. The genes used to compare expression profiles of the cell types were as follows: Pluripotency (*Oct4*, *nanog*, and *Sox 2*), cell differentiation [teratocarcinoma-derived growth factor 1 (*TGDF1*), *DNM3TB*, gamma-aminobutyric acid (*GABA*) A receptor, beta 3 (*GABRB3*), growth differentiation factor-3 (*GDF3*), *Lefty*, *Nodal*, and semaphorin 3a (*Sema3a*)], and neural lineage specification [paired box gene 6 (*Pax6*), *Nestin*, oligodendrocyte transcription factor 2 (*Olig2*), glial fibrillary acidic protein (*GFAP*), and synaptophysin]. TaqMan primer sequence information is proprietary (www.appliedbiosystems.com).

Animal care and surgical procedures

Adult (6–8 weeks old) *shiverer* mice (*shi/shi*) (C3Fe.SWV-Mbp-Shi/J; The Jackson Laboratory) were used for transplantation experiments. The *shiverer* mouse has a deletion in the myelin basic protein (*MBP*) gene resulting in poorly compacted and sparse CNS myelin [26]. The *shiverer* mouse lacks the endogenous capacity for remyelination and is well suited for evaluating the myelinating potential of transplanted cell types [7,27]. To characterize the neural differentiation pattern of the NSC populations in vivo, we transplanted the cell types into the spinal cords of *shiverer* mice. Before surgery, mice were immunosuppressed by the administration of cyclosporine A (20 mg/kg, 10 mg/kg after 14 days) in their drinking water 48 h before receiving transplants and continuing until the animals were sacrificed. Mice were also treated with Minocycline (50 mg/kg) beginning 48 h before surgery for 10 days for both its immunosuppressive properties, the inhibition of microglial activation [28], and its modest neuroprotective properties [29,30] to limit potential injury due to the injection procedure. Under

TABLE 1. PRIMARY ANTIBODIES

Primary antibody name	Manufacturer	Cell lineage	Antigen description	Working dilution
APC (CC-1)	Calbiochem	Mature oligodendrocytes (in vivo)	Expressed on oligodendrocyte cell bodies	1:40
β III Tubulin (Tuj 1)	Chemicon	Neurons	Neuronal-specific tubulin	1:200
CNPase		Mature oligodendrocytes	Myelin protein, expressed by immature and mature oligodendrocytes	1:100
GFAP	Chemicon	Astrocytes, O2A cells	Intermediate filament expressed by astrocytes	1:500
MAP-2 α,β	Sigma	Neurons	Neuronal-specific cytoskeletal protein	1:100
MBP	Sternberger Monoclonal II	Mature oligodendrocytes	Myelin protein expressed by mature, myelinating oligodendrocytes	1:1,000
Nestin	Chemicon	Neural progenitors	Type IV intermediate filament expressed by undifferentiated neural stem cells	1:100
NF200	Chemicon	Axons	Neuronal-specific intermediate filament expressed in axons	1:100
Olig2	Chemicon and Abcam	Oligo lineage undifferentiated NSCs/NPCs/Motor neuron progenitors	bHLH transcription factor expressed by cells in all stages of the oligodendrocyte lineage	1:1,000:1,000
PDGFR α	Santa Cruz Biotech	Immature oligodendrocytes	Receptor protein marker for oligodendrocyte precursor cells.	1:40

APC, adenomatous polyposis coli; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; PDGFR α , platelet-derived growth factor alpha; NSC, neural stem cells; NPCs, neural precursor cells.

isoflurane anesthesia (1%–2% with 1:1 mixture of O₂/N₂O) a T6 laminectomy was performed to expose the spinal cord, and cells were injected using a pulled glass micropipette attached to a 5 μ L Hamilton syringe. Cells were transplanted bilaterally adjacent to the midline dorsal vein at a depth of 0.5 mm. All cell types were prepared for transplantation by dissociation and resuspension at a concentration of 50,000 cells/ μ L in SFM. The injection volume was 2 μ L per site and cells were injected at a rate of 0.5 μ L/min by a computer-controlled syringe pump (World Precision Instruments). The cell injection procedure was well tolerated and did not result in any detectable locomotor or behavioral deficits in the mice. No behavioral assessment was conducted on transplanted animals. For this study the *shiverer* model was employed as a means to directly compare the neural differentiation pattern of dNSCs and aNPCs and not as a method to assess or compare therapeutic potential.

Immunohistochemistry and histology

Six weeks after cell transplantation, animals were administered an overdose of sodium pentobarbital (Somnotol) and transcardially perfused with ice-cold PBS followed by 4% PFA in PBS (pH 7.4). A 1 cm section of spinal cord centered on the injection site was removed and 14- μ m-thick longitudinal spinal cord cryostat sections were prepared. Before staining, nonspecific antibody binding was blocked by incubation in PBS containing 1% BSA, 5% NGS, 5% Nonfat

milk powder, and 0.25% Triton X-100 for 1 h at room temperature. Primary antibodies (Table 1) were applied in the same solution overnight at 4°C and slides were washed before application of the appropriate fluorescently conjugated secondary antibodies. Nuclei were counterstained by the use of VECTASHIELD mounting medium with DAPI (Vector Labs).

Cell counting and imaging methods

Quantification of in vitro differentiation was conducted by cell counts to determine the proportion of cells expressing a given cell marker. For each lineage marker, 12 separate wells were quantified for each primary antibody with 3 separate cell differentiation experiments conducted from distinct starting ESC populations. Using a Nikon C1 confocal microscope, 9 nonoverlapping images were collected in each culture slide well using a 20 \times objective. For each well, the number of positively stained cells and the total number of cells present were counted by DAPI-stained nuclei, to determine the proportion of cells expressing a given marker. Cells were counted using ImageJ software (<http://rsbweb.nih.gov/ij/>). For cell transplantation experiments, the differentiation pattern was quantified by determining the proportion of total YFP-positive transplanted cells that expressed each lineage marker. Histological sections used for each marker were separated by a minimum of 100 μ m to eliminate double counting of cells. Nonoverlapping 60 \times

images were acquired of the all transplanted cells detected in each cord section. Cells staining positively for each lineage marker were counted and expressed as a proportion of the total transplanted YFP-positive cells present.

Statistical analysis

Cell count data are presented using descriptive statistics. The differences between groups were analyzed using z-tests for comparing proportions with the Bonferroni correction applied for multiple comparisons. Real-time quantitative PCR data was presented as a log fold expression change relative to the control. The differences between groups were analyzed using one-way ANOVA and the post hoc Student's *t*-test, and *P* values of <0.05 were considered statistically significant. Statistical analysis was performed using Microsoft Excel. Data are presented as means \pm standard error of the mean or as proportions \pm standard error of the proportion.

Results

Production of clonal neurospheres from ESCs through the default pathway

The dissociation of ES colonies into a single-cell solution under colony-forming assay conditions (10 cells/ μ L) in defined SFM containing LIF led to the formation of floating spherical colonies of cells, termed primitive neurospheres and derived clonally from ESCs as previously reported [14,15]. Primitive neurospheres appeared as dense cellular accumulations with a smooth outer surface when observed under a phase-contrast microscope. Primitive neurospheres could be passaged into SFM containing FGF2 and heparin (no LIF) to produce definitive neurospheres, clonally derived from dNSCs. Neurospheres derived from dNSCs could also be passaged repeatedly in agreement with previous findings [14,15] (Fig. 1A). The gross morphology of definitive, dNSC-derived neurospheres was distinct from primitive neurospheres in that they did not have a smooth outer surface and individual cell profiles could be seen. Definitive neurospheres appear similar in morphology to adult brain-derived neurospheres colonies (Fig. 1B–D).

In vitro comparative gene expression profiling

The genes examined fell into 3 broad categories. (1) Genes involved in the maintenance of pluripotency [*Oct4*, *nanog*, and *SRY* (sex determining region Y)-box 2 (*Sox2*)], (2) genes involved in maintenance of an undifferentiated cell state (*TDGF1*, *DNM3TB*, *GABRB3*, *GDF3*, and *Sema3a*), and (3) genes involved in neural differentiation (*Pax6*, *Nestin*, *Olig2*, and *synaptophysin*). The expression level of the genes in undifferentiated ESCs was used as the baseline for calculating relative expression levels. We hypothesized that the expression pattern observed in pNSCs would be somewhat similar to that seen in ESCs, whereas the dNSCs would show a pattern of expression more similar to aNPCs. For the pluripotency genes, we observed a downregulation of expression of *Oct4*, *nanog*, and *Sox2* in pNSCs versus ESCs and a downregulation of the genes in dNSCs and aNPCs for *nanog* and *Oct4*. *Sox2* was expressed at similar levels in all

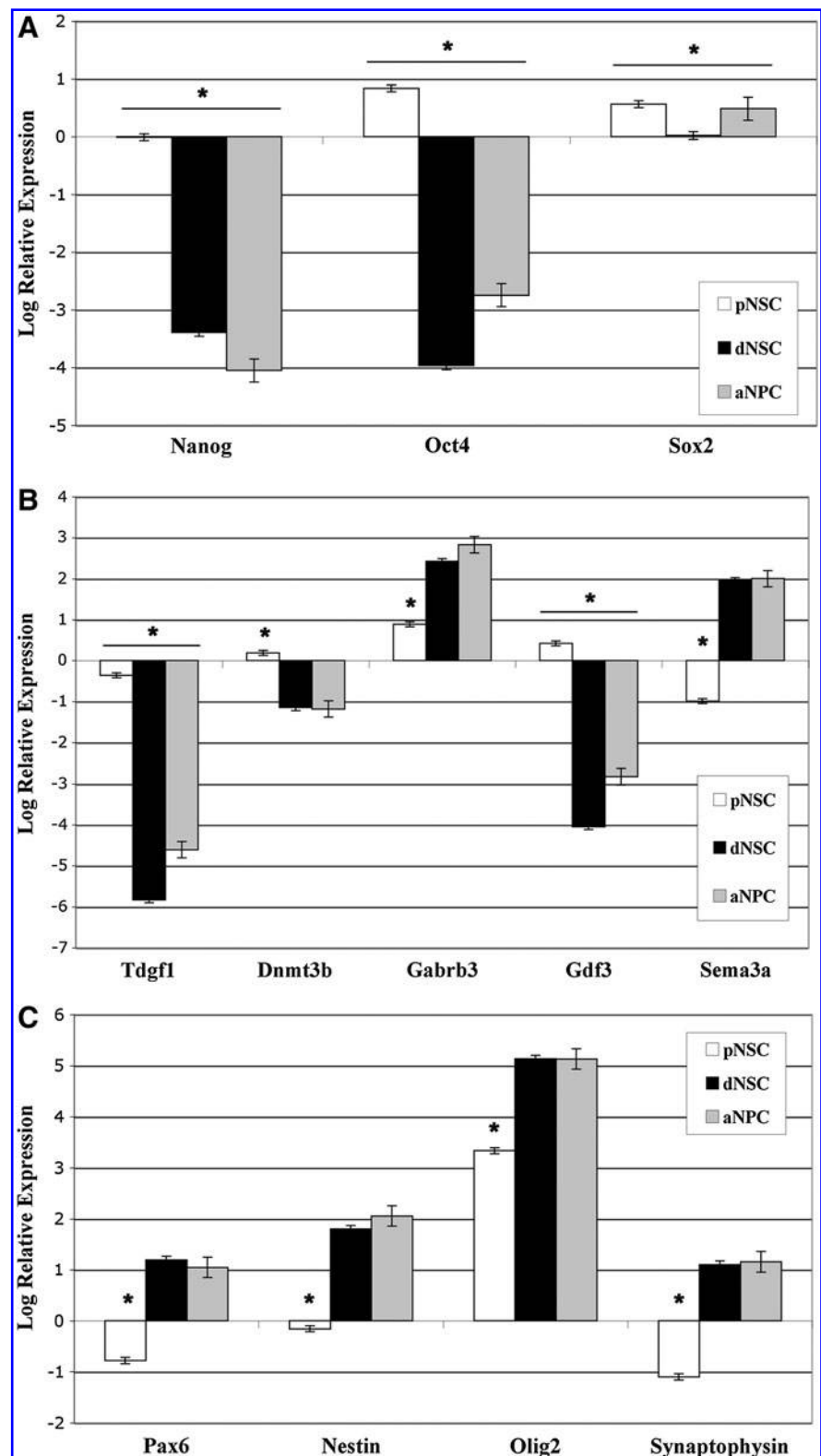
cell types likely due to its role in both pluripotency [31] and neural stem cells [32] (Fig. 2A). For genes related to maintenance of an undifferentiated cell state, we observed a similar pattern of expression for all the genes in dNSCs and aNPCs, where again the pNSCs were more similar to ESCs (Fig. 2B). For the genes related to neural differentiation, we found a similar level expression in dNSCs and aNPCs for all 4 genes examined. *Olig2* was the only neural gene that was upregulated in pNSCs versus the ESC control (Fig. 2C). Based on the gene expression patterns observed, the default-derived dNSC and aNPCs had a similar expression profile, whereas the pNSC had a profile that was intermediate between ESCs and more committed NSCs.

Characterization of neural differentiation patterns in vitro

The differentiation of aNPCs resulted in the production of all 3 cell types of the neural lineage—neurons, astrocytes, and oligodendrocytes—as previously reported [1,21,24]. Astrocyte lineage cells, as indicated by expression of GFAP, were the major cell type observed with $65\% \pm 1.5\%$ of cells staining positively for GFAP. Oligodendrocytes accounted for $18\% \pm 1.2\%$ of the cells as indicated by expression of *Olig2*, which is expressed by cells in all stages of the oligodendrocyte lineage. *PDGFR α* was expressed by $13\% \pm 1.1\%$ of cells identifying them as oligodendrocyte progenitors or precursor cells [33,34]. Neurons represented $7\% \pm 0.8\%$ of differentiated aNPCs as indicated by their expression of the neuronal marker beta-III tubulin. Approximately $1\% \pm 0.3\%$ of aNPCs continued to express the neural stem/progenitor cell marker *nestin* (Fig. 3 and Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/scd). Based on the panel of neural lineage markers, the total percentage of cells accounted for by the 3 neural lineages and *nestin*-expressing undifferentiated NSCs was 91%. Therefore, 9% of cells remained undefined based on this assay.

Based on previous findings [14] and the similar gene expression pattern we observed in dNSCs and aNPCs, we hypothesized that the differentiation of dNSC neurospheres colonies would produce a similar pattern as that observed for aNPCs for the panel of neural antigens. The observed pattern contained all 3 neural lineages, and astrocytes accounted for the majority of cells observed with $61\% \pm 1.5\%$ of cells expressing GFAP. In contrast to aNPCs, differentiated dNSCs produced more neurons than oligodendrocytes with $11\% \pm 1.0\%$ of cells expressing beta III-tubulin, $6.1\% \pm 0.7\%$ expressing *Olig2*, and only $2\% \pm 0.5\%$ expressing *PDGFR α* . *Nestin* expression was maintained in $9.5\% \pm 0.9\%$ of dNSCs after 1 week in differentiating conditions (Fig. 4 and Supplementary Fig. S2). Based on these data, the total percentage of differentiated dNSCs defined by our assay was 89%, leaving 11% of the cells undefined. While GFAP-expressing cells were the majority of the cells produced by both aNPCs and dNSCs in vitro, we found that the dNSCs produced a significantly higher proportion (2-tail $P < 0.05$) of presumed neuronal lineage cells, as judged by beta III-tubulin expression, and a significantly lower proportion of *Olig2*-positive cells relative to aNPCs. The subset of the *Olig2*-expressing cell population that expresses the oligodendrocyte progenitor marker *PDGFR α* was also significantly lower in the dNSC

FIG. 2. Gene expression relative to undifferentiated ES colonies in neurospheres generated from pNSCs, dNSCs, and aNPCs. Relative quantification by real-time reverse transcription–polymerase chain reaction analysis of expression of selected genes. **(A)** Pluripotency genes: pNSC-derived neurospheres maintain expression of pluripotency genes expressed in ESCs. Neurospheres generated by dNSCs and aNPCs downregulate Oct4 and Nanog but maintain expression of Sox2. **(B)** For selected genes involved in the maintenance of an undifferentiated state; dNSC and aNPC-generated neurospheres display similar pattern of gene expression. **(C)** For a selection of neural genes dNSC and aNPC-generated neurospheres display a similar pattern of gene expression that is not observed in spheres generated from pNSCs. * $P < 0.05$. POU5F1, Oct 4, octamer-binding transcription factor 4; Sox2, SRY (sex determining region Y)-box 2; TDGF1, teratocarcinoma-derived growth factor 1; DNMT3b, DNA (cytosine-5)-methyltransferase 3 beta; GABRB3, gamma-aminobutyric acid (GABA) A receptor, beta 3; GDF3, growth differentiation factor 3; sema3a, semaphorin 3A; aNPC, adult neural precursor cell.



group versus the aNPC (Fig. 5). dNSCs produced mature oligodendrocytes; some cultures contained CNPase-expressing cells with the morphology of fully matured in vitro oligodendrocytes [33] (Fig. 6).

Based on the ES-like pattern of gene expression observed for pNSCs, it was hypothesized that the differentiation

pattern of pNSCs in vitro would not produce a pure population of neural lineage cells. This was observed with the differentiation pattern of these cells appearing somewhat similar to that of ES differentiation through EB formation with many distinct morphologies that did not express any neural markers for which they were stained

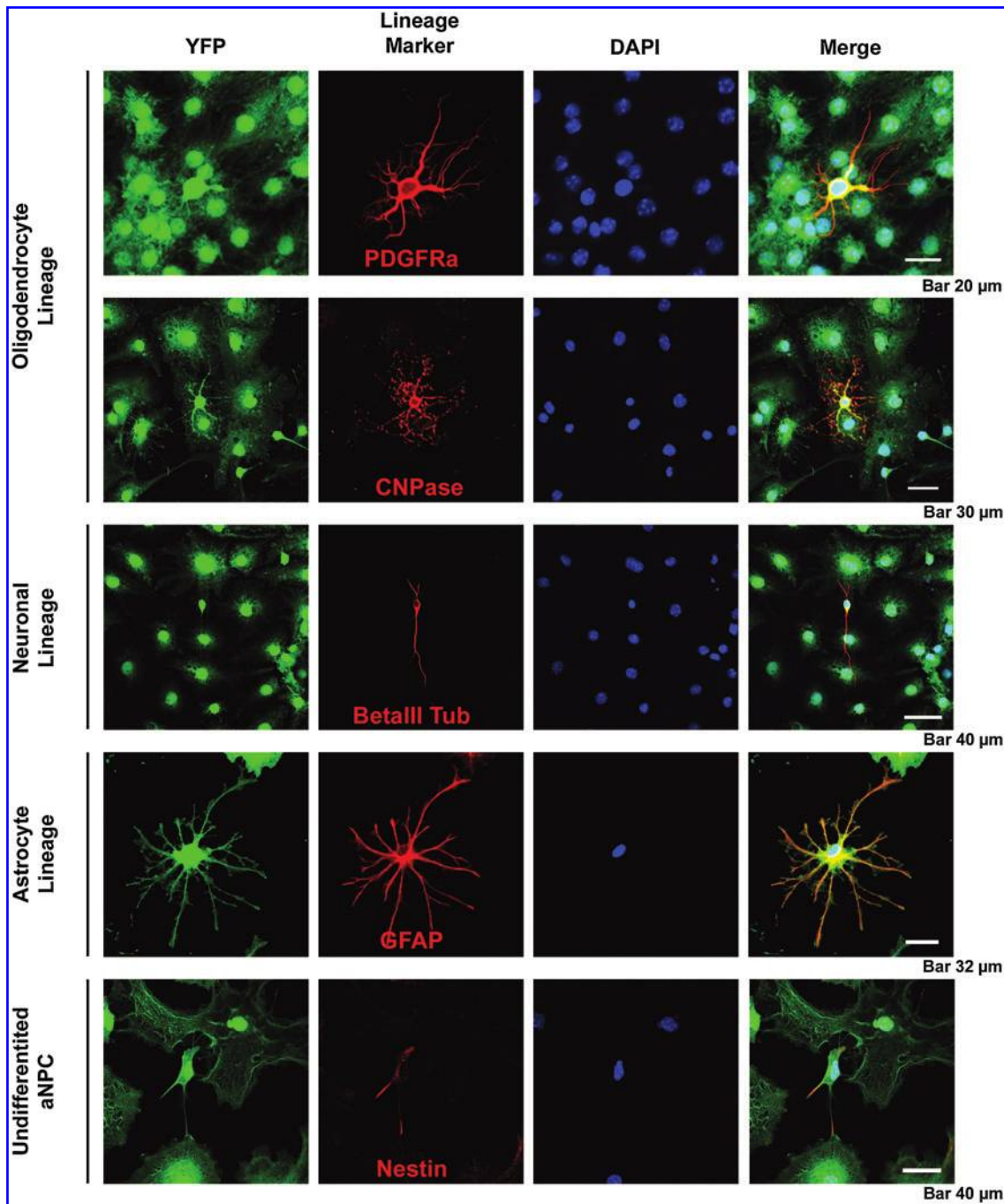


FIG. 3. Characterizing the pattern of in vitro neural differentiation of aNPCs. All 3 neural lineage cell types are produced by aNPC-generated neurosphere colonies when differentiated for 1 week in 1% FBS. Lineages were identified using immunocytochemical identification of the following lineage antigens: PDGFR α and CNPase for the oligodendrocyte lineage, β III tubulin for the neuronal lineage, GFAP for the astrocyte lineage, and nestin expression was used to identify undifferentiated aNPCs. FBS, fetal bovine serum; PDGFR α , platelet-derived growth factor alpha; GFAP, glial fibrillary acidic protein.

(data not shown). Despite this, pNSC differentiation did result in many cells expressing neural markers. Quantification revealed that the most prevalent neural marker observed was nestin, which was seen in $39\% \pm 0.8\%$ of cells. $5\% \pm 1.5\%$ of cells expressed GFAP, and approximately 1% of cells expressed Olig2 or PDGFR α . In areas of high cell density, beta-III tubulin-expressing cells extended long axonal-like processes that formed complex networks

(Fig. 7). Accurately counting cells in these areas, using the DAPI-stained nuclei counting technique, was nearly impossible due to dense cell accumulations. Individual cells expressing beta-III tubulin that could be identified accounted for approximately 1% of pNSCs. Based on the panel of markers used, 46% of cells in 1 week differentiated pNSC cultures were defined, leaving 54% of cells undefined (Fig. 5).

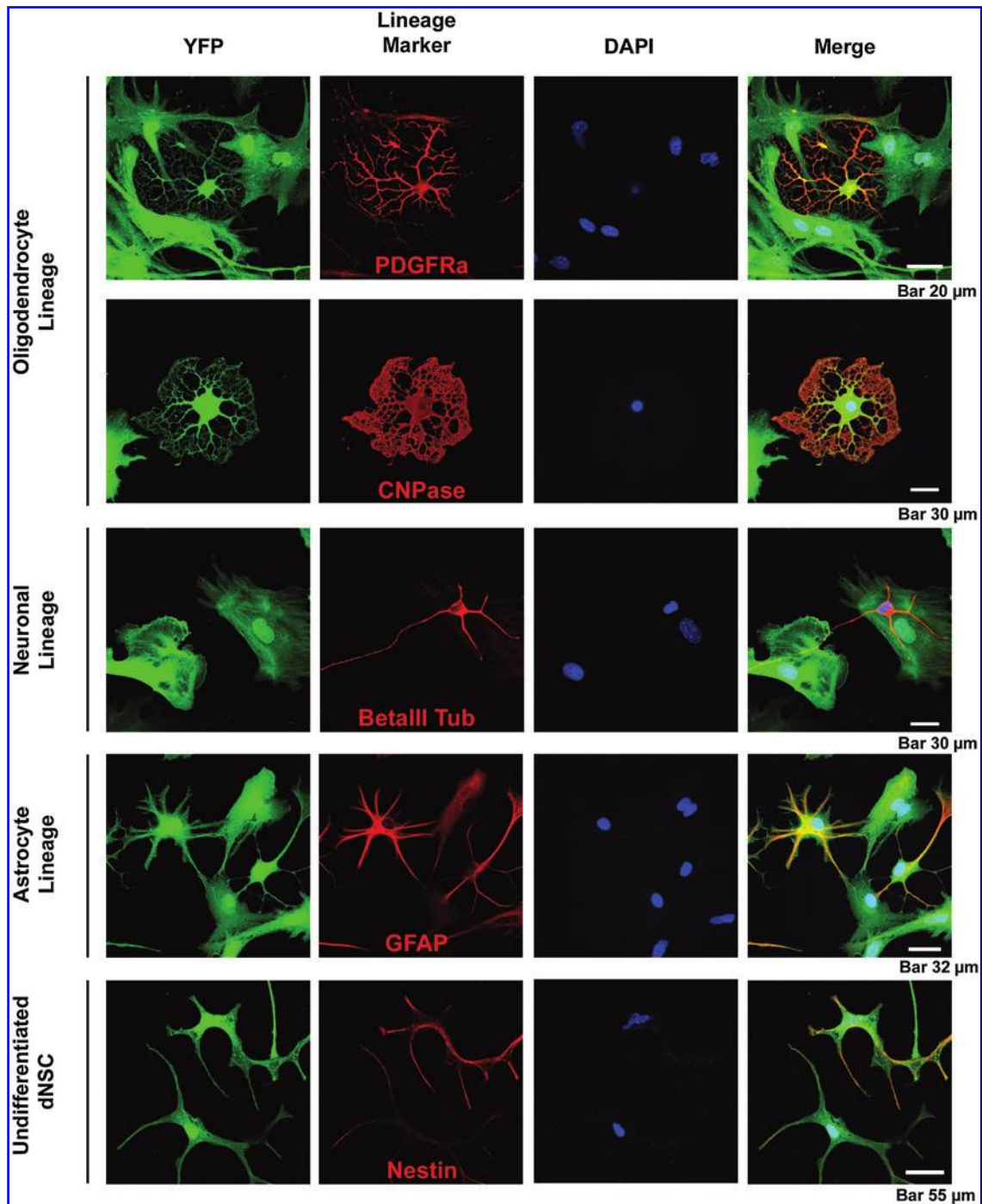


FIG. 4. Characterizing the pattern of in vitro neural differentiation of dNSCs. All 3 neural lineage cell types are produced by dNSC-generated neurosphere colonies when differentiated for 1 week in 1% FBS. Lineages were identified using immunocytochemical identification of the following lineage antigens: PDGFR α and CNPase for oligodendrocyte lineage cells, β III tubulin for neuronal lineage, GFAP for the astrocyte lineage, and nestin expression was used to identify undifferentiated dNSCs. DAPI, 4',6-diamidino-2-phenylindole; YFP, yellow fluorescent protein.

Differentiation after transplantation into the dysmyelinated spinal cord

The findings that the properties of dNSCs in vitro were similar to aNPCs were encouraging, and suggested that the two cell types might differentiate similarly when transplanted into the dysmyelinated spinal cords of *shiverer* mice. In

agreement with previous reports from our laboratory [27], the lack of compact myelin and MBP expression was confirmed in the *shiverer* mice used for this study (Supplementary Fig. S3).

The results of our transplantation of aNPCs confirmed previous findings that aNPCs transplanted into white matter of the adult CNS can survive and integrate well into the host spinal cord parenchyma and differentiate into oligodendro-

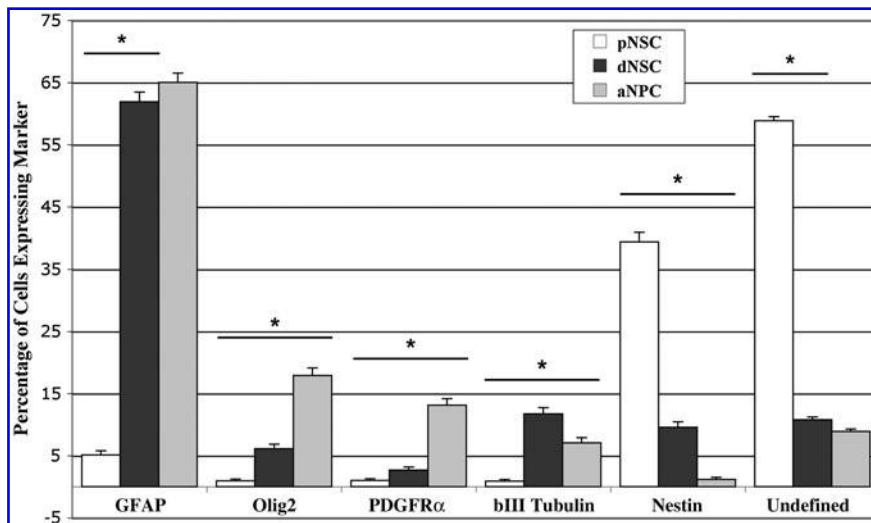


FIG. 5. Quantification of the in vitro neural differentiation pattern of pNSCs, dNSCs, and aNPCs after 1 week in nonspecific differentiation conditions (1% FBS). Quantification of the neural differentiation pattern as proportions of cells reveals that the major cell type produced by both dNSCs and aNPCs after in vitro differentiation is GFAP-expressing astrocyte lineage cells. For pNSCs, after 1 week the largest proportion did not express any of the neural antigens used and the next largest group was nestin-expressing, undifferentiated neural stem or progenitor cells. * $P < 0.05$.

cytes with multiple processes that express MBP (Figs. 8A and 9A) [1,27]. We quantified the pattern of differentiation of the aNPCs and observed that based on the neural antigens we used the majority of YFP-expressing transplanted cells differentiated into the oligodendrocyte lineage, as indicated by the expression of Olig2 by $47\% \pm 2.7\%$, adenomatous polyposis coli (APC) by $34\% \pm 2.7\%$, and PDGFR α by $15\% \pm 3.7\%$ of transplanted cells. Note that the percentage of cells expressing APC or PDGFR α is a subpopulation of the Olig2-positive proportion. We also detected YFP-expressing cells that expressed GFAP ($3.4\% \pm 3.0\%$), microtubule-associated protein 2 (MAP2; $3.8\% \pm 3.6\%$), and rare cells that maintained expression of nestin ($0.7\% \pm 0.38\%$) (Supplementary Figs. S4 and S5). When single transplanted cells were examined at high magnification using confocal microscopy, some of the cells displayed an oligodendrocyte-like morphology with multiple extended processes that aligned with host axons and stained positively for MBP (Fig. 9A).

In comparison to the transplantation of aNPCs, the transplantation of pNSCs did not result in successful integration of grafted cells in the spinal cord. Similar to the observations from the in vitro differentiation, a large number of YFP-expressing transplanted pNSCs failed to express any markers for which they were assessed. pNSC-transplanted regions contained areas where transplanted cell types were similar to those observed with aNPCs, although also had areas where highly irregular cell growth were observed,

containing cells with an epithelial-like morphology. These cells did not express nestin, which is expressed in neuroepithelial cells (data not shown). The presence of these heterotomas was associated with locomotor impairments in the animals, and the transplantation of pNSC was not pursued further in light of these findings.

In contrast to the results obtained with pNSCs, dNSCs survived and integrated into the spinal cord white matter in a similar manner as NPCs (Figs. 8B, C and 9B). Transplanted dNSCs extended multiple processes, associated with host NF200-expressing axons, and expressed MBP consistent with the myelination of axons previously reported [27]. Individual transplanted dNSCs, viewed under high magnification confocal microscopy, had a similar morphology to that observed for aNPCs. Some extended multiple processes that aligned with host axons and expressed MBP, suggesting that they were mature, myelin-forming oligodendrocytes (Fig. 9B). Quantification of the differentiation pattern of transplanted dNSCs revealed that the majority of cells differentiated into the oligodendrocyte lineage (Figs. 10 and 11). This finding is similar to that of transplanted aNPCs, although the proportion of oligodendrocytes was significantly higher (two-tailed $P < 0.05$) for dNSCs than for aNPCs. Cells expressing Olig2 accounted for $58\% \pm 4.5\%$ of YFP-positive transplanted cells. Oligodendrocyte lineage-specific markers were also expressed by transplanted dNSCs, with $42 \pm 6.2\%$ expressing APC and $19 \pm 3.0\%$ expressing PDGFR α . GFAP was

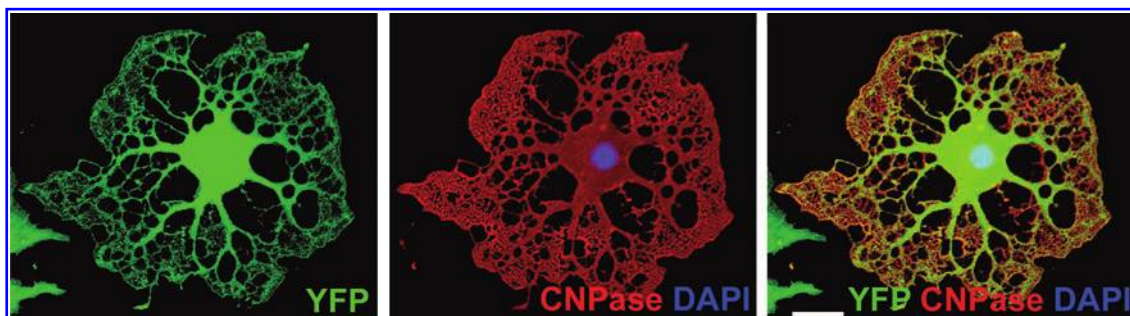


FIG. 6. Mature oligodendrocyte derived from ESCs using the default pathway of neural differentiation. A CNPase-expressing oligodendrocyte derived from ESCs using the default pathway of neural induction. CNPase is a marker for mature oligodendrocytes in vitro. Scale bar represents 20 μm .

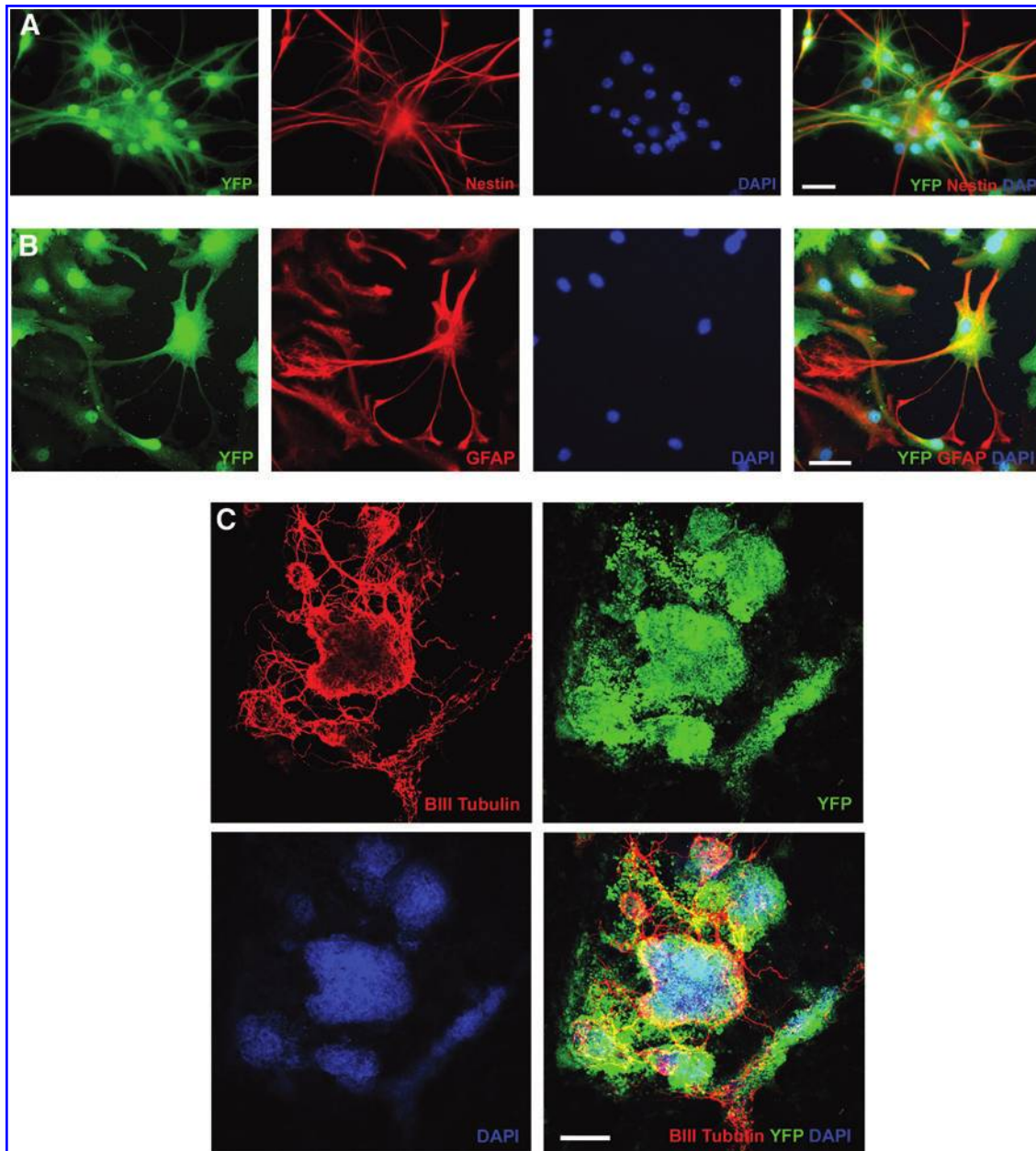


FIG. 7. Characterizing the pattern of in vitro neural differentiation of pNSCs. The differentiation of pNSCs for 1 week in 1% FBS produced some cells that expressed neural markers. **(A)** Nestin-expressing pNSCs-derived cells present after 1 week in differentiating conditions. **(B)** pNSC-derived astrocyte lineage cells. **(C)** Differentiation of pNSCs resulted in dense accumulations of cells that elaborated networks of β III tubulin-expressing fibers. Scale bar represents 20 μ m in **(A)** and **(B)**, 200 μ m in **(C)**.

expressed by $4.2 \pm 0.4\%$ and the neuronal marker MAP2 was detected in $3.1 \pm 0.4\%$ of cells. Nestin-expressing cells accounted for $3.0 \pm 0.4\%$ of all cells (Supplementary Fig. S6). Based on these data and using the expression of Olig2, GFAP, MAP2, and nestin to represent the neural lineages and undifferentiated NSCs, we found that 68% of cells of transplanted dNSCs were accounted for by our differentiation assay. The same summation of the proportional data for transplanted aNPCs showed 56%, a significantly lower percentage (2-tailed $P < 0.05$), of cells were defined by the neural differentiation assay. Stated another way, 32% of transplanted dNSCs and 44% of transplanted aNPCs were unaccounted for based on the neural antigen panel used for

quantification (Fig. 11). Previous work by our laboratory involving the quantification of the in vivo differentiation of aNPCs has observed similar portions of transplanted cell types failing to express neural markers [1].

Discussion

In this report we have demonstrated that the default pathway for production of NSCs from ESCs [14,15,35] is a promising method to produce neurosphere generating, clonal NSCs with properties similar to that of adult brain-derived NSCs (Table 2). Most important, we also have shown the first in vivo differentiation of NSCs (dNSCs), derived from ESCs

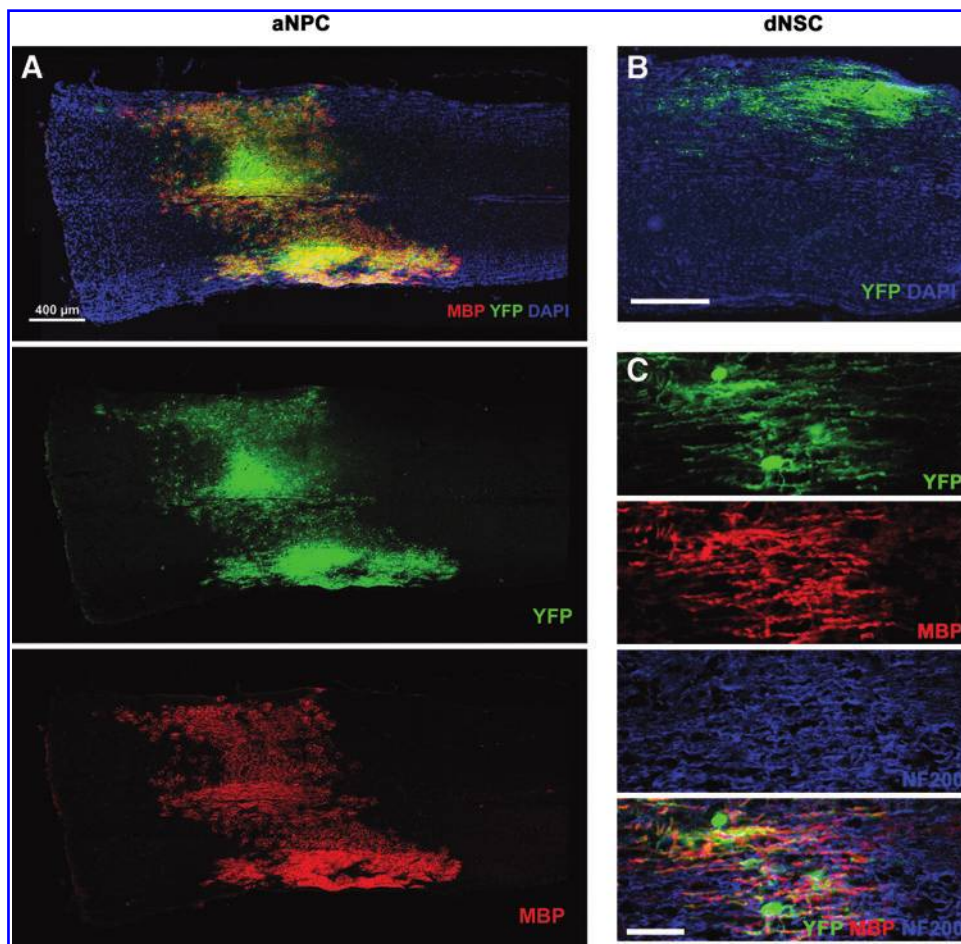


FIG. 8. Transplantation of ES-derived definitive neurospheres and adult brain-derived neurospheres into the spinal cord of *shiverer* mice. (A) Transplanted aNPCs survive, integrate into white matter, and differentiate into cells with multiple processes that express MBP in the *shiverer* spinal cord. (B) Transplanted dNSCs also survive and integrated well into the *shiverer* spinal cord. (C) Transplanted dNSCs expressed extended multiple processes that expressed MBP and associated with NF200-expressing host axons. Scale bars represent 400 μm in (A) and (B) and 20 μm in (C). MBP, myelin basic protein.

through the default mechanism, into the oligodendrocyte lineage. We found that the dNSCs produced through the default pathway are similar to aNPCs in their neurosphere morphology, gene expression profile, in vitro pattern of neural differentiation, and in vivo pattern of neural differentiation after transplantation into the dysmyelinated spinal cord. In the *shiverer* spinal cord, both transplanted dNSCs and aNPCs differentiated primarily into the oligodendrocyte lineage, integrated well into the spinal cord parenchyma, and expressed MBP, suggesting the formation of myelin. Further investigation of the potential of transplanted dNSCs to produce myelinating oligodendrocytes in relevant animal models is required to determine their therapeutic potential. However, our findings are in parallel with previous studies from our laboratory [27] that showed that MBP-expressing cells derived from transplanted aNPCs can ensheath multiple axons and form compact myelin—as shown using electron microscopy—that led to improved axonal conduction as confirmed using in vivo electrophysiology. The therapeutic potential of these myelinating cells was demonstrated in a rodent model of spinal cord injury where they have been found to significantly enhance functional recovery [1,36].

The default pathway and development

The production of NSCs from ESCs using the default pathway of neural induction is a powerful tool to generate large quantities of neural lineage cells. Although the precise

details of the lineage relationship between the pNSC, dNSC, and aNPC remain unclear, it is likely that neural induction through the default mechanism largely recreates the neural lineage progression as it functions in normal development [37]. The default model was originally proposed based on findings in *Drosophila* [38] and *Xenopus* [39,40], but subsequent findings using murine [14,15,19] and human ESCs [41,42] have supported this model. There are striking similarities between the sequence of LIF dependence in pNSCs followed by FGF2 dependence in dNSCs observed after default neural induction in ESCs to the populations of LIF responsive neural lineage cells between embryonic days 5.5 and 7.5 (E5.5–7.5), which give rise to an FGF2-dependent cell population after E8.5 in the developing mouse embryo [37]. When looking forward to the production of cell populations for clinical application, the use of cell differentiation techniques that recreate or activate naturally occurring developmental programs is likely a valuable strategy in producing the safe and predictable cell populations.

The removal of LIF and passage of pNSCs to the dNSC results in significant cell death—LIF has been shown to function as a pro-survival factor in this culture system [14]—and this has been suggested as a limitation of ES neural differentiation protocols based on the default model [43]. While cell death is prominent after LIF withdraw once a population of dNSCs is established, the cells can be propagated in a similar fashion to aNPCs allowing for the production of large numbers of cells. It

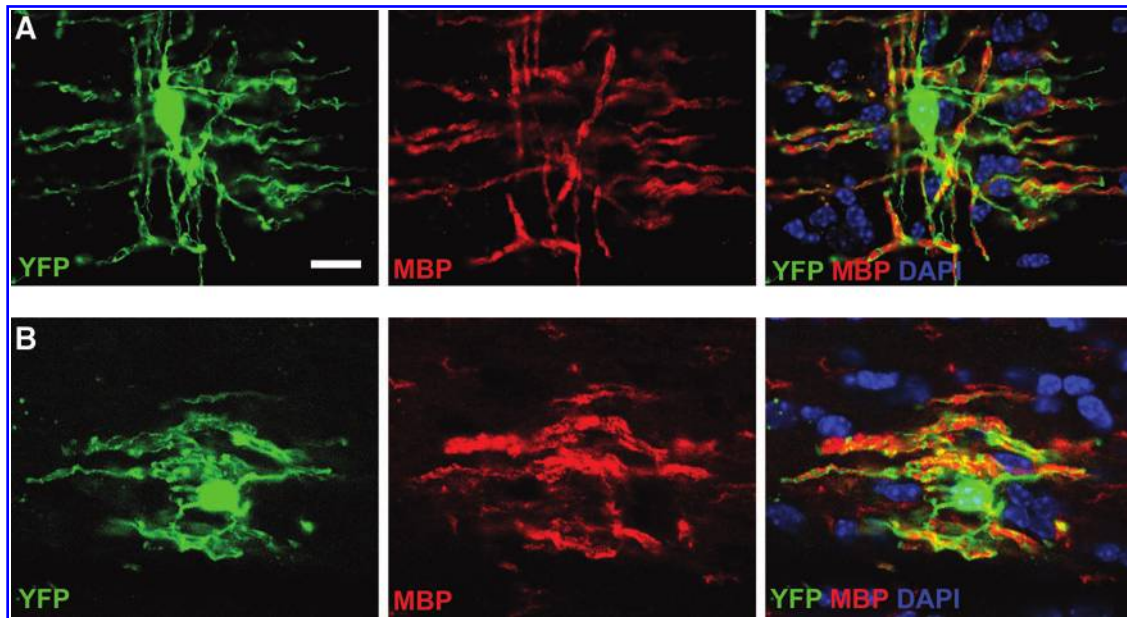


FIG. 9. Transplantation of ESC-derived definitive neurospheres and adult brain-derived neurospheres into the spinal cord of *shiverer* mice produces some cells that express MBP and have the morphologies of oligodendrocytes. Transplanted aNPCs and dNSCs differentiate into cells with many properties of oligodendrocytes. Both aNPCs (A) and dNSCs (B) produce a cell type that expresses multiple processes that align with host axons and express MBP. Scale bar represents 15 μ m.

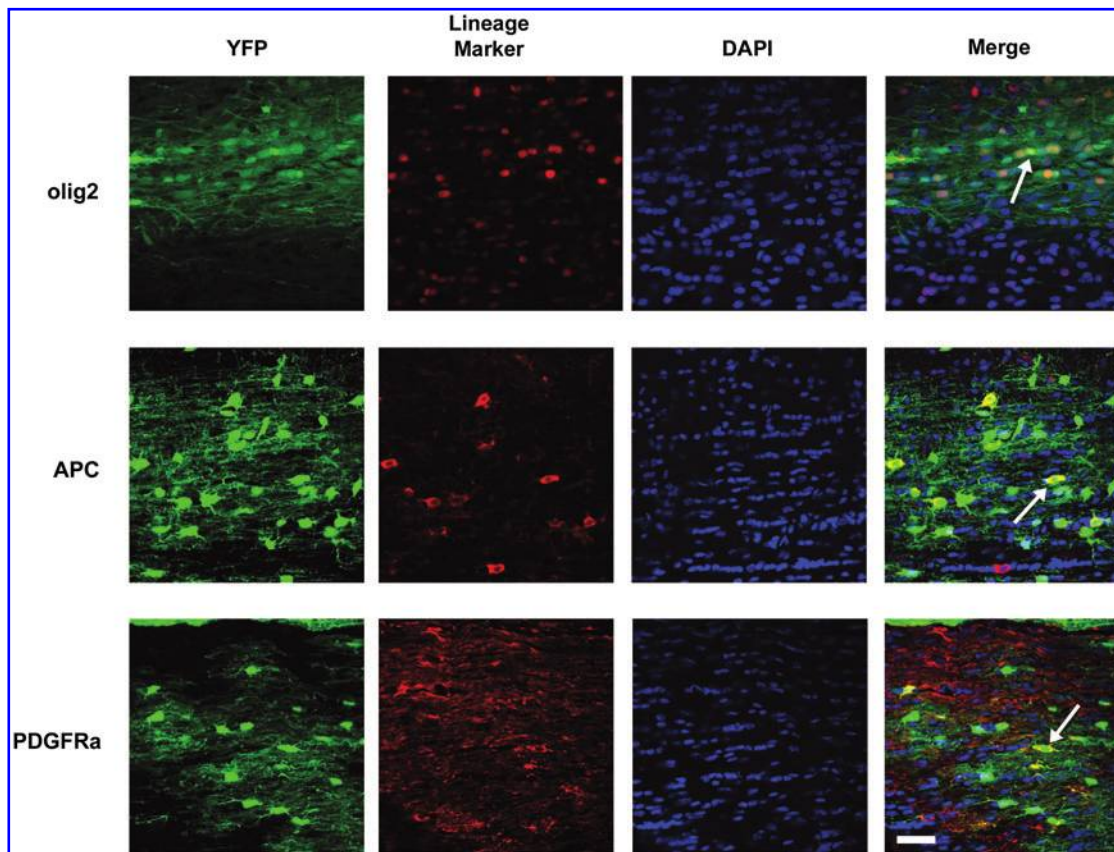


FIG. 10. Sample counting fields for oligodendrocyte lineage markers used in quantification of the *in vivo* neural differentiation of pattern of dNSCs 6 weeks after transplantation into the spinal cord of *shiverer* mice. Typical image fields used for cell quantification are shown for transplanted dNSCs stained for the oligodendrocyte lineage markers Olig2, APC, and PDGFR α . Arrowheads indicate cells in which the lineage marker is observed to colocalize with YFP of the transplanted dNSCs. Scale bar represents 15 μ m.

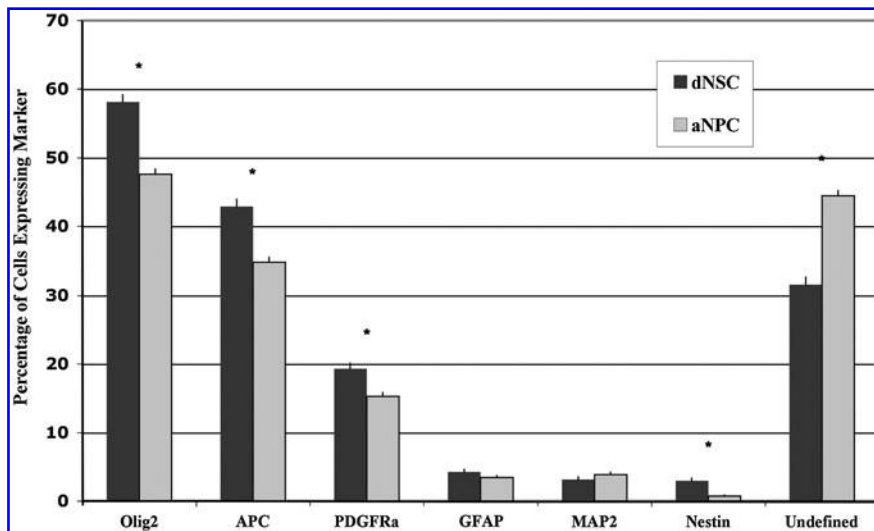


FIG. 11. Neural differentiation pattern of dNSCs and aNPCs after transplantation into the dysmyelinated spinal cord of *shiverer* mice. Quantification of the neural differentiation pattern as proportions of surviving transplanted cells reveals that the major cell type produced by both dNSCs and aNPCs when transplanted into the *shiverer* spinal cord is the oligodendrocyte. Olig2-expressing oligodendrocyte lineage cells accounted for a significantly higher proportion of transplanted dNSCs than aNPCs. The Olig2-expressing cell population consisted largely of APC-expressing mature oligodendrocytes and PDGFR α -expressing oligodendrocyte progenitor cells. Astrocytes and neuronal lineage cells made up a small proportion of

surviving differentiating cells for both dNSCs and aNPCs. The proportion of nestin-expressing transplanted cells was small for both cell types, but a significantly higher proportion of nestin-expressing cells were observed for surviving transplanted dNSCs relative to aNPCs. * $P < 0.05$.

also has been demonstrated recently that when cultured at more physiological relevant oxygen concentrations than standard culture conditions, the cell death observed for both pNSC and dNSC decreases and expression of neural lineage genes such as *Sox1*, *Sox2*, nestin, and β III tubulin is actually enhanced [35]. Therefore, the initial cell death and low yield of pNSCs and dNSCs may not be a significant limitation to the production of dNSCs with this method.

Definitive NSCs as an alternative to adult neural precursors

Based on our findings, the dNSC and aNPC are similar with respect to their gene expression profile and neural differentiation pattern both in vitro and in vivo in a model of CNS dysmyelination. After a week of nonspecific differentiating conditions in vitro, the astrocyte lineage—as defined by GFAP expression—accounted for the largest proportion

TABLE 2. SUMMARY OF DEFINITIVE NEURAL STEM CELL AND ADULT FOREBRAIN SUBPENDYMAL ZONE PROPERTIES

	dNSC	aNPC
Cell source	Embryonic stem cells (pluripotent cell populations).	Subependymal region of adult CNS (eg, forebrain subventricular zone).
Production method	Neural induction through the default pathway to produce neurospheres colonies (serum-free, minimal culture conditions in presence of LIF, followed by FGF after passage).	Isolation and dissociation followed by culture in serum-free conditions with EGF and FGF2 to produce neurospheres colonies.
In vitro neural differentiation (1% FBS, 7 days)	All 3 neural lineages produced: Astrocytes: 62% Neurons: 12% Oligodendrocytes: 6%	All 3 neural lineages produced: Astrocytes: 65% Neurons: 7% Oligodendrocytes: 18%
Transplantation into <i>shiverer</i> mouse spinal cord	Transplanted cells: Survive integrate into host CNS Many develop multiple processes, associate with host axons, and express MBP suggestive of remyelination	Transplanted cells: Survive Integrate into host CNS Many develop multiple processes, associate with host axons, and express MBP suggestive of remyelination.
In vivo neural differentiation (6 weeks in <i>shiverer</i> spinal cord)	Transplanted cells mainly differentiation into the oligodendrocyte lineage: Oligodendrocytes: 58% Astrocytes: 4% Neurons: 3%	Transplanted cells mainly differentiation into the oligodendrocyte lineage: Oligodendrocytes: 48% Astrocytes: 3% Neurons: 4%

FGF, fibroblast growth factor; LIF, leukemia inhibitory factor; CNS, central nervous system; EGF, epidermal growth factor; FBS, fetal bovine serum.

of differentiated cells produced by both cell types. However, the dNSCs were observed to be more neurogenic than aNPCs under identical differentiation conditions *in vitro*. Neurons were the second most frequent cell type observed for differentiated dNSCs, whereas the oligodendrocyte lineage (as defined by Olig2 or PDGFR α expression) was more prevalent than the neuronal lineage (as defined by β III-tubulin expression) in the aNPCs. This discrepancy might be explained by the normal developmental progression of the mammalian CNS in which neurogenesis dominates the early period of development, whereas gliogenesis increases with developmental age. In the developing CNS, neurogenesis is initiated at E9 and the first committed OPCs are not present until approximately E12 [44–47]. Since the dNSC population can be considered as a developmentally more primitive cell type than the aNPC, based on its lack of EGF-dependence and being a small (<10) number of passages removed from ESCs, it is possible that the observed difference in neuronal versus oligodendrocyte differentiation is a reflection of the normal developmental time course. The comparison of long-term passaged dNSCs where EGF is included in the cultures to replicate the conditions used to culture aNPCs would help address this question, as it is unknown whether dNSCs *in vitro* can develop the EGF dependence, which is observed in NSCs in developing mouse embryo [37]. Notch signaling has been demonstrated to likely play an important role in this process although it is unclear if exogenous notch activation is required or if the development of EGF dependence does occur spontaneously in ES-derived dNSCs *in vitro* although at a very low rate [37]. Despite the differences observed during the 1-week *in vitro* differentiation in the more clinically relevant, 6-week differentiation period within the *shiverer* spinal cord there was no observed preference for dNSCs to adopt a neural fate above that of aNPCs (Fig. 11). However, despite the similar proportion of cells adopting a neuronal fate (3.1% and 3.8% for dNSCs and aNPCs, respectively) there were differences observed in the *in vivo* neural differentiation pattern between the 2 cell types. While Olig2-expressing cells were the predominant cell type produced by both dNSCs and aNPCs, dNSCs did produce a significantly higher proportion of Olig2-positive cells (58% and 47%, respectively) and a significantly lower proportion of undefined cells (32% and 44%, respectively). When we examine the largest component of the Olig2-positive cell population—the oligodendrocyte lineage—we see larger proportions of both PDGFR α -expressing oligodendrocyte progenitors (19% and 15%, respectively) and mature oligodendrocytes (42% and 34%, respectively) for the progeny of the dNSCs relative to the aNPCs. We also observed enhanced proportional nestin expression (3% and 0.7%, respectively) for the progeny of dNSCs versus aNPCs. These differences could be a result of staining and counting errors or small sample size, although they also may be the result of enhanced oligodendrocyte differentiation being an inherent property of dNSCs relative to aNPCs. This is an important question that must be addressed in future work to determine the optimal cell type for remyelinating cell therapy.

The dysmyelinated spinal cord of the *shiverer* mouse can be considered an instructive or permissive cellular niche that likely promotes transplanted NSCs into preferentially adopting an oligodendrocyte fate. The *shiverer* has been shown to possess an excess of NG2-expressing oligodendro-

cyte progenitors [48] and it is likely that the signals resulting in this proliferative response in the NG2 cell population could also affect the fate of transplanted cells. The specific signals (and their source) that underlie this proliferative response by oligodendrocyte progenitors in the *shiverer* remain to be fully elucidated. Previous work has demonstrated that oligodendrocyte progenitors do respond to demyelination by mounting a proliferative response [49]. This response may be induced by the signaling molecules GRO-1 [50] and neuregulin (glial growth factor) [51], which may be released by astrocytes and neurons, respectively. Both molecules have been demonstrated to act as mitogens, to promote survival, and to inhibit differentiation of oligodendrocyte progenitors. It is likely that these signals (or others) resulting in the observed proliferative response in the NG2 cell population would also affect the fate of transplanted NSCs. Previous studies have also suggested the instructive nature of the niche as transplanted cells from ESC [52], fetal [53], and adult sources [27] have been shown to produce myelin-forming oligodendrocytes after transplantation and in other instances have demonstrated large-scale cell replacement and even rescue of the *shiverer* phenotype [7,8]. Here, we employed the *shiverer* mouse model not to directly assess the therapeutic potential of the dNSCs but as a means to compare the differentiation pattern of dNSCs to aNPCs. Our evidence for the similar behavior of the dNSC and aNPC suggests that the dNSC may be of equal therapeutic benefit as the aNPCs, which have been shown to function to replace lost cells throughout adult life in the brain [21,23,24] and have been demonstrated to have a marked ability to integrate well into CNS parenchyma when transplanted into the adult CNS—a finding that has been observed again in the experiments reported here. The observation of terminally differentiated oligodendrocytes both *in vitro* and after transplant into the *shiverer* mouse shows that differentiation based on the default pathway of neural induction is a promising method for producing clonal NSCs and their derivatives from a pluripotent cell population. Most important, this method could be of high clinical relevance by providing therapeutic NSC populations from a pluripotent source population and by minimizing the risk of contamination of unwanted cell lineages by obviating the need for EB formation.

Addressing the undefined proportion after differentiation

Quantification of the pattern of neural differentiation both after *in vitro* differentiation and after transplantation in the *shiverer* mouse resulted in portions of the differentiated cell population being unaccounted for by the panel of neural antigens we used. The undefined proportion of the cells was difference of 1 minus the sum of the proportions for the markers Olig2, GFAP, beta-III tubulin or MAP2, and nestin, which represent the 3 neural lineages and undifferentiated NSCs. Technical staining and counting errors likely contribute to the undefined proportion of the cell populations, particularly for the quantification of the *in vivo* pattern of differentiation as it was not uncommon for adjacent transplanted cells with very similar morphologies to not express the same marker. In such cases, one cell was correctly counted as negative for the given marker. MBP was a very reliable indicator of mature oligodendrocytes but could not

be used to for quantification since the pattern of oligodendrocyte myelination results in adjacent myelin segments on an axon coming from multiple nearby oligodendrocytes, which makes quantification very difficult and inaccurate. Previous work has demonstrated that aNPCs do not differentiate into Schwann cells (SCs) based on expression of the p75 neurotrophin receptor and the peripheral myelin marker P0 [1,27]. Future studies should address the possibility of SC differentiation, although based on the morphology of transplanted aNPCs and dNSCs observed, we hypothesize that dNSCs do not produce SCs under the differentiating conditions examined in this report. Accounting for an undefined proportion of labeled cells is further complicated by cell markers not being unique to a specific cell lineage. For example, Olig2 is typically considered an oligodendrocyte lineage marker [33] but is also expressed by NSCs and in neurospheres (data not shown), has been shown to have a role in white matter astrocyte development [54], and is expressed in the pMN progenitor cell population that yields motor neurons and oligodendrocytes [55]. While further work is required to fully characterize the progeny of both aNPCs and dNSCs, our findings here suggest that dNSCs might be of equal, if not greater, therapeutic potential as aNPCs.

Conclusions

This work has demonstrated that neural induction in ESCs through the default mechanism can produce a cell type—the definitive NSC—that is a potential substitute for NSC derived from the forebrain subependymal cells of the adult brain. Application of this approach using human ESC or induced pluripotent stem cells (iPSCs) is a very promising strategy to produce NSC for research or potential therapeutic applications such as remyelination after CNS injury. Further investigation of the cells produced through the default pathway when applied to human ES or iPS cells is required to fully determine the potential and clinical relevance of this neural induction method.

Acknowledgments

The authors would like to thank Desiree Schut and Jian Wang of the Fehlings laboratory and Sue Runciman and Brenda Coles of the van der Kooy laboratory for their assistance with cell culture and Behzad Ahzad for his assistance with animal care. The authors would also like to thank Dr. Soheila Karimi-Abdolrezaee for her technical expertise and guidance while conducting these experiments and Lauren Fehlings and Madeleine O'Higgins for their assistance with the article. This work was made possible by funding from the MS Society of Canada (studentship to J.W.R.), the Canadian Institutes of Health Research (operating grants to M.G.F.), and the Krembil Foundation (M.G.F. and D.V.K.).

Author Disclosure Statement

No competing financial interests exist.

References

- Karimi-Abdolrezaee S, E Eftekharpour, J Wang, CM Morshead and MG Fehlings. (2006). Delayed transplantation of adult neural precursor cells promotes remyelination and functional neurological recovery after spinal cord injury. *J Neurosci* 26:3377–3389.
- Keirstead HS, G Nistor, G Bernal, M Totoiu, F Cloutier, K Sharp and O Steward. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 25:4694–4705.
- McDonald JW, XZ Liu, Y Qu, S Liu, SK Mickey, D Turetsky, DI Gottlieb and DW Choi. (1999). Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat Med* 5:1410–1412.
- Brustle O, KN Jones, RD Learish, K Karam, K Choudhary, OD Wiestler, ID Duncan and RD McKay. (1999). Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* 285:754–756.
- Fricke-Gates RA and MA Gates. (2010). Stem cell-derived dopamine neurons for brain repair in Parkinson's disease. *Regen Med* 5:267–278.
- Lamba D, M Karl and T Reh. (2008). Neural regeneration and cell replacement: a view from the eye. *Cell Stem Cell* 2:538–549.
- Windrem MS, MC Nunes, WK Rashbaum, TH Schwartz, RA Goodman, G McKhann 2nd, NS Roy and SA Goldman. (2004). Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. *Nat Med* 10:93–97.
- Windrem MS, SJ Schanz, M Guo, GF Tian, V Washco, N Stanwood, M Rasband, NS Roy, M Nedergaard, et al. (2008). Neonatal chimerization with human glial progenitor cells can both remyelinate and rescue the otherwise lethally hypomyelinated shiverer mouse. *Cell Stem Cell* 2:553–565.
- Nistor GI, MO Totoiu, N Haque, MK Carpenter and HS Keirstead. (2005). Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. *Glia* 49:385–396.
- Murry CE and G Keller. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132:661–680.
- Bain G, D Kitchens, M Yao, JE Huettner and DI Gottlieb. (1995). Embryonic stem cells express neuronal properties *in vitro*. *Dev Biol* 168:342–357.
- Lee H, GA Shamy, Y Elkabetz, CM Schofield, NL Harrision, G Panagiotakos, ND Succi, V Tabar and L Studer. (2007). Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. *Stem Cells* 25:1931–1939.
- Sadowski D, ME Kiel, M Apicella, AG Arriola, CP Chen and R McKinnon. (2010). Teratogenic potential in cultures optimized for oligodendrocyte development from mouse embryonic stem cells. *Stem Cells Dev* 19:1343–1353.
- Smukler SR, SB Runciman, S Xu and D van der Kooy. (2006). Embryonic stem cells assume a primitive neural stem cell fate in the absence of extrinsic influences. *J Cell Biol* 172:79–90.
- Tropepe V, S Hitoshi, C Sirard, TW Mak, J Rossant and D van der Kooy. (2001). Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 30:65–78.
- Munoz-Sanjuan I and AH Brivanlou. (2002). Neural induction, the default model and embryonic stem cells. *Nat Rev Neurosci* 3:271–280.
- Ying QL, M Stavridis, D Griffiths, M Li and A Smith. (2003). Conversion of embryonic stem cells into neuroectodermal

- precursors in adherent monoculture. *Nat Biotechnol* 21: 183–186.
18. Hemmati-Brivanlou A and D Melton. (1997). Vertebrate embryonic cells will become nerve cells unless told otherwise. *Cell* 88:13–17.
 19. Kamiya D, S Banno, N Sasai, M Ohgushi, H Inomata, K Watanabe, M Kawada, R Yakura, H Kiyonari, et al. (2011). Intrinsic transition of embryonic stem-cell differentiation into neural progenitors. *Nature* 470:503–509.
 20. Nagy A, J Rossant, R Nagy, W Abramow-Newerly and JC Roder. (1993). Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 90:8424–8428.
 21. Reynolds BA and S Weiss. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175:1–13.
 22. Coles-Takabe BL, I Brain, KA Purpura, P Karpowicz, PW Zandstra, CM Morshead and D van der Kooy. (2008). Don't look: growing clonal versus nonclonal neural stem cell colonies. *Stem Cells* 26:2938–2944.
 23. Reynolds BA and S Weiss. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 255:1707–1710.
 24. Morshead CM, BA Reynolds, CG Craig, MW McBurney, WA Staines, D Morassutti, S Weiss and D van der Kooy. (1994). Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* 13:1071–1082.
 25. Schmittgen TD and KJ Livak. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108.
 26. Chernoff GF. (1981). Shiverer: an autosomal recessive mutant mouse with myelin deficiency. *J Hered* 72:128.
 27. Eftekharpour E, S Karimi-Abdolrezaee, J Wang, H El Behairy, C Morshead and MG Fehlings. (2007). Myelination of congenitally dysmyelinated spinal cord axons by adult neural precursor cells results in formation of nodes of Ranvier and improved axonal conduction. *J Neurosci* 27:3416–3428.
 28. Festoff BW, S Ameenuddin, PM Arnold, A Wong, KS Santacruz and BA Citron. (2006). Minocycline neuroprotects, reduces microgliosis, and inhibits caspase protease expression early after spinal cord injury. *J Neurochem* 97:1314–1326.
 29. Wells JE, RJ Hurlbert, MG Fehlings and VW Yong. (2003). Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. *Brain* 126:1628–1637.
 30. Teng YD, H Choi, RC Onario, S Zhu, FC Desilets, S Lan, EJ Woodard, EY Snyder, ME Eichler and RM Friedlander. (2004). Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury. *Proc Natl Acad Sci USA* 101:3071–3076.
 31. Kashyap V, NC Rezende, KB Scotland, SM Shaffer, JL Persson, LJ Gudas and NP Mongan. (2009). Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. *Stem Cells Dev* 18:1093–1108.
 32. Graham V, J Khudyakov, P Ellis and L Pevny. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* 39:749–765.
 33. Grinspan J. (2002). Cells and signaling in oligodendrocyte development. *J Neuropathol Exp Neurol* 61:297–306.
 34. Grinspan JB and B Franceschini. (1995). Platelet-derived growth factor is a survival factor for PSA-NCAM+ oligodendrocyte pre-progenitor cells. *J Neurosci Res* 41:540–551.
 35. Clarke L and D van der Kooy. (2009). Low oxygen enhances primitive and definitive neural stem cell colony formation by inhibiting distinct cell death pathways. *Stem Cells* 27:1879–1886.
 36. Karimi-Abdolrezaee S, E Eftekharpour, J Wang, D Schut and MG Fehlings. Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord. *J Neurosci* 30:1657–1676.
 37. Hitoshi S, RM Seaberg, C Kosciak, T Alexson, S Kusunoki, I Kanazawa, S Tsuji and D van der Kooy. (2004). Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling. *Genes Dev* 18:1806–1811.
 38. Wharton KA, RP Ray and WM Gelbart. (1993). An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* 117:807–822.
 39. Wilson PA and A Hemmati-Brivanlou. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* 376:331–333.
 40. Zimmerman LB, JM De Jesus-Escobar and RM Harland. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86:599–606.
 41. Schulz TC, SA Noggle, GM Palmarini, DA Weiler, IG Lyons, KA Pensa, AC Meedeniya, BP Davidson, NA Lambert and BG Condie. (2004). Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. *Stem cells* 22:1218–1238.
 42. Vallier L, D Reynolds and RA Pedersen. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev Bio* 275:403–421.
 43. Chambers SM, CA Fasano, EP Papapetrou, M Tomishima, M Sadelain and L Studer. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 27:275–280.
 44. Calver AR, AC Hall, WP Yu, FS Walsh, JK Heath, C Betsholtz and WD Richardson. (1998). Oligodendrocyte population dynamics and the role of PDGF *in vivo*. *Neuron* 20:869–882.
 45. Billon N, C Jolicoeur, QL Ying, A Smith and M Raff. (2002). Normal timing of oligodendrocyte development from genetically engineered, lineage-selectable mouse ES cells. *J Cell Sci* 115:3657–3665.
 46. Hardy R and R Reynolds. (1991). Proliferation and differentiation potential of rat forebrain oligodendroglial progenitors both *in vitro* and *in vivo*. *Development* 111:1061–1080.
 47. Pringle NP, S Guthrie, A Lumsden and WD Richardson. (1998). Dorsal spinal cord neuroepithelium generates astrocytes but not oligodendrocytes. *Neuron* 20:883–893.
 48. Bu J, A Banki, Q Wu and A Nishiyama. (2004). Increased NG2(+) glial cell proliferation and oligodendrocyte generation in the hypomyelinating mutant shiverer. *Glia* 48: 51–63.
 49. Di Bello IC, MR Dawson, JM Levine and R Reynolds. (1999). Generation of oligodendroglial progenitors in acute inflammatory demyelinating lesions of the rat brain stem is associated with demyelination rather than inflammation. *J Neurocytol* 28:365–381.
 50. Wu Q, RH Miller, RM Ransohoff, S Robinson, J Bu and A Nishiyama. (2000). Elevated levels of the chemokine GRO-1

- correlate with elevated oligodendrocyte progenitor proliferation in the jimpy mutant. *J Neurosci* 20:2609–2617.
51. Canoll PD, JM Musacchio, R Hardy, R Reynolds, MA Marchionni and JL Salzer. (1996). GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors. *Neuron* 17:229–243.
52. Liu S, Y Qu, TJ Stewart, MJ Howard, S Chakraborty, TF Holekamp and JW McDonald. (2000). Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. *Proc Natl Acad Sci USA* 97:6126–6131.
53. Mitome M, HP Low, A van den Pol, JJ Nunnari, MK Wolf, S Billings-Gagliardi and WJ Schwartz. (2001). Towards the reconstruction of central nervous system white matter using neural precursor cells. *Brain* 124:2147–2161.
54. Cai J, Y Chen, WH Cai, EC Hurlock, H Wu, SG Kernie, LF Parada and QR Lu. (2007). A crucial role for Olig2 in white matter astrocyte development. *Development* 134:1887–1899.
55. Ligon KL, SP Fancy, RJ Franklin and DH Rowitch. (2006). Olig gene function in CNS development and disease. *Glia* 54:1–10.

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Received for publication April 29, 2011

Accepted after revision May 20, 2011

Prepublished on Liebert Instant Online May 23, 2011

This article has been cited by:

1. Ryan P. Salewski , Josef Buttigieg , Robert A. Mitchell , Derek van der Kooy , Andras Nagy , Michael G. Fehlings . The Generation of Definitive Neural Stem Cells from PiggyBac Transposon-Induced Pluripotent Stem Cells Can Be Enhanced by Induction of the NOTCH Signaling Pathway. *Stem Cells and Development*, ahead of print. [[Abstract](#)] [[Full Text HTML](#)] [[Full Text PDF](#)] [[Full Text PDF with Links](#)] [[Supplemental material](#)]