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Human Embryonic Stem Cells Differentiate into Oligodendrocytes in High Purity and Myelinate After Spinal Cord Transplantation

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ABSTRACT Human embryonic stem cells (hESCs) demonstrate remarkable proliferative and developmental capacity. Clinical interest arises from their ability to provide an apparently unlimited cell supply for transplantation, and from the hope that they can be directed to desirable phenotypes in high purity. Here we present for the first time a method for obtaining oligodendrocytes and their progenitors in high yield from hESCs. We expanded hESCs, promoted their differentiation into oligodendroglial progenitors, amplified those progenitors, and then promoted oligodendroglial differentiation using positive selection and mechanical enrichment. Transplantation into the *shiverer* model of dysmyelination resulted in integration, differentiation into oligodendrocytes, and compact myelin formation, demonstrating that these cells display a functional phenotype. This differentiation protocol provides a means of generating human oligodendroglial lineage cells in high purity, for use in studies of lineage development, screening assays of oligodendroglial-specific compounds, and treating neurodegenerative diseases and traumatic injuries to the adult CNS.

INTRODUCTION

One of the greatest challenges facing hESC research is the derivation of high-purity lineages from pluripotent hESCs. The directed differentiation of embryonic stem cells into high-purity fates would provide novel and powerful tools for studying the cellular and molecular mechanisms of developmental transitions, and for generating donor cells for transplantation therapies. The derivation of high-purity lineages would render hESCs useful in clinical strategies, as it is likely that differentiated cells would result in greater engraftment efficiency, enhanced clinical efficacy, decreased probability of spontaneous differentiation into undesired lineages, and reduced risk of teratoma formation, compared with the transplantation of undifferentiated hESCs (Keirstead, 2001; Loebel et al., 2003).

The decision of a pluripotent cell to progress toward a lineage during development requires the concerted effort of both inductive and inhibitory signals, and the ability of cells to respond to such signals. Although some progress has been made in identifying growth factors that play a role in early differentiation of hESCs (Schuldiner et al., 2000; Pera et al., 2004), the directed differentiation of hESCs to a highly purified

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neural cell type has thus far not been demonstrated. Most efforts to generate homogeneous populations have been based on selection of the desired phenotype based on expression of lineage-specific markers or transgenes, or ectopic expression of lineage-specific transcription factors, approaches which suffer from poor selection efficacy and the fact that genetic manipulations may impair the ability of the cells to undergo the full repertoire of lineage differentiation.

hESC research benefits from two decades of mouse stem cell research, which has provided us with methods to differentiate mouse stem cells into several clinically relevant neural (Deacon et al., 1998; Brustle et al., 1999; Lee et al., 2000; Liu et al., 2000; Kim et al., 2003) and non-neural (Wiles and Keller, 1991; Klug et al., 1996; Soria et al., 2000) cell types. The generation of functional derivatives from mouse embryonic stem cells has been reported, despite the inability to generate high-purity populations. Differentiated dopamine-producing neurons incorporate into the striatum and improve behavioral performance following transplantation into mouse models of Parkinson's disease (Kim et al., 2002; Nishimura et al., 2003), and mouse embryonic stem cell-derived motor neurons survive and differentiate following transplantation into the embryonic chick spinal cord (Wichterle et al., 2002).

Although hESCs have been directed to differentiate into multipotent neural precursors (Zhang et al., 2001; Reubinoff et al., 2001; Carpenter et al., 2001), their differentiation to a purified neural population has thus far not been demonstrated. Here we outline a method for the differentiation of oligodendrocytes and their progenitors in high yield from hESCs, without the use of genetic modification. Transplantation into the *shiverer* mouse resulted in integration, oligodendrocyte differentiation and compact myelin formation, demonstrating that these cells display a functional phenotype. These studies demonstrate that the directed differentiation of hESCs to a high-purity neural population is possible, and provide for the first time the means to generate functional human oligodendroglial lineage cells in large quantity for research and clinical application.

MATERIALS AND METHODS

Cellular Differentiation

The H7 hESC line at passage 32 was obtained from Geron Corporation; the origin and derivation of this cell line is described by Thomson et al. (1998). Media was replaced daily for the duration of hESC expansion and every other day during the differentiation protocol. This study was conducted 8 times, using cells from passage 32–39. Cells were expanded on feeder-free Matrigel (BD Bioscience, Lexington, KY) substrate in hESC growth media according to published protocols (Carpenter et al., 2001). The hESC growth media consisted of conditioned media generated by an irradiated layer of mouse fibroblasts grown for 24 h in the following media: knockout DMEM (Gibco-Invitrogen, Carlsbad, CA), 10% knockout serum replacement (Gibco-Invitrogen), 4 ng/ml human recombinant basic fibroblast growth factor (bFGF; Gibco-Invitrogen), 1% MEM nonessential amino acid solution (Gibco-Invitrogen), 1 mM L-glutamine (Gibco-Invitrogen), and 3.5 μl/500 ml β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). This conditioned media was supplemented with 8 ng/ml human recombinant bFGF at the time of feeding. Cultures were passaged every week for the duration of hESC expansion.

To encourage the formation of neurospheres, cell colonies were dissociated from adherent substrate with collagenase IV (Gibco-Invitrogen) and placed in ultra-low binding six-well plates (Fisher Scientific, Pittsburgh, PA) for 2 days in a transition media consisting of 50% hESC growth media (above), and 50% glial restriction media (GRM). GRM consisted of DMEM: F12, B27 supplement (Gibco-Invitrogen), 25 μg/ml insulin (Sigma-Aldrich), 6.3 ng/ml progesterone (Sigma-Aldrich), 10 μg/ml putrescin (Sigma-Aldrich), 50 ng/ml sodium selenite (Sigma-Aldrich), 50 μg/ml holotransferin (Sigma-Aldrich), and 40 ng/ml triiodothyroidin (Sigma-Aldrich). The transition media was supplemented for 1 day with 4 ng/ml bFGF and 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich).

Cells were then exposed to transition media supplemented with 2 ng/ml bFGF, 20 ng/ml EGF (Sigma-Aldrich), and 10 μM/ all-*trans*-retinoic acid (RA) (Sigma-Aldrich) in DMSO (Sigma-Aldrich) for 1 day. This media was then replaced with 100% GRM supplemented with 20 ng/ml EGF (Sigma-Aldrich) and 10 μM/ all-*trans*-RA in DMSO for a further 7 days. Up to this point cultures were fed daily; after this point cultures were fed every other day.

RA was then omitted from media for the duration of the differentiation protocol. Cells were exposed to GRM supplemented with 20 ng/ml EGF for 18 days. At this point (day 28), cultures were exposed to a mechanical selection procedure that involved transferring cultures to 1:30 growth factor-reduced Matrigel-coated plates overnight. Cultures were then gently shaken to dislodge all but the yellow spheres (which preferentially adhere to the plates), and the media was replaced with fresh GRM supplemented with 20 ng/ml EGF for a further 7 days of growth on 1:30 growth factor reduced Matrigel coated plates. Cultures were then (day 35) exposed to trypsin-EDTA (Gibco-Invitrogen) for 2–5 min to dislodge the spheres and plated on 1:30 Matrigel substrate in 75–225-cm^2 vented-cap flasks (Fisher Scientific), and cultured for 1 week in GRM supplemented with 20 ng/ml EGF. During the last 5 days of this step, some cultures were exposed to 10 μM BrdU (Sigma-Aldrich) and transplanted into the shiverer mouse model of demyelination (below). Other cultures were exposed at the end of the differentiation protocol (day 42) to culture conditions which promoted their terminal differentiation (below). In some cases cells were maintained in GRM supplemented with 20 ng/ml EGF for 3, 6, or 8 weeks to expand their

numbers before terminal differentiation. Cultures were passaged every week.

The size of spheres was measured at 4× magnification using an optical ruler. Measurements were taken from 10 randomly sampled spheres from each of three separate tissue culture wells, and averaged. The percentage of yellow spheres was calculated by dividing the number of yellow spheres by the total number of spheres in three randomly chosen 6.25-mm^2 fields within each of three separate tissue culture wells, and averaged.

For terminal differentiation and immunocytochemical staining, cells were plated at low density on poly-L-lysine-coated (Sigma-Aldrich) and human laminin-coated (Sigma-Aldrich) four-chamber imaging slides (Fisher Scientific). Media was changed every other day.

Immunocytochemical Staining

Cultures were fixed 1 week later by exposure to 4% paraformaldehyde (Fisher Scientific) in PB for 10 min. Nuclear staining was conducted by exposing cultures to Mayer's hematoxylin for 10 min and blued in ammonia water for 10 min. Immunocytochemical staining was performed using standard protocols. Imaging slides were blocked with 20% serum for 30 min at room temperature. Primary antiserum (mouse anti-O4, 1:50, Chemicon, Temecula, CA; rabbit anti-GalC, 1:200, Chemicon; mouse anti-RIP, 1:100, Chemicon; rabbit anti-NG-2, 1:100, Chemicon; rabbit anti-SOX10, 1:200, Chemicon; rabbit anti-BMP4, 1:100, Chemicon; rabbit anti-Olig1, 1:200, Chemicon; mouse anti-A2B5, 1:100, Chemicon; mouse anti-Tuj1, 1:200, CRP, Denver, PA; rabbit anti-Pax6, 1:100, CRP; rabbit anti-cow glial fibrillary acidic protein (GFAP), 1:500, DAKO, Denmark; mouse anti-SSEA4 supernatant, 1:5, a kind gift from Geron Corporation) were diluted in 4% serum and applied to imaging chambers overnight at 4°C. Imaging chambers were rinsed three times with PBS, incubated for 30 min in 20% serum, and biotinylated antisera (rabbit anti-goat, goat anti-mouse or goat anti-rabbit biotinylated IgG H+L, 1:200, Vector Laboratories, Burlingame, CA) was applied and incubated for 1 h at room temperature. Chambers were washed three times in PBS and incubated for 30 min in a methanol 3% and 0.3% hydrogen peroxide solution, and for 1 h in ABC solution (Vector) at room temperature. Chambers were rinsed three times in PBS, and DAB substrate solution (Vector) was used for visualization. Cell quantification was conducted using an Olympus AX-80 light microscope with a 20× objective. The percentage of immunopositive cells was determined by dividing the total number of immunopositive cells by the total number of hematoxylin positive cells in each imaging chamber, and averaging the results from three imaging chambers per marker.

Transplantation and Electron Microscopy

Cells were concentrated to a final density of 100,000 cells per μl. Transplants were conducted in 3–4-week-old Shiverer mice (Jackson Laboratory, Bar Harbor, ME), which have a life span of approximately 10 weeks, allowing 6 weeks for cellular differentiation and myelination. Animals were anesthetized with Avertin (0.6 ml/20 g) and injected in the center of the dorsal column at T9 with 4 μl of cells in suspension over 10 min using a pulled glass micropipette of 60-μm outside diameter with polished tip (n = 8). The needle was removed from the cord 5 min after injection of the cells was complete. Control animals received injections of 4 μl knock-out DMEM in a similar manner (n = 8). All animals received daily injections of 10 mg/kg cyclosporine (Ben-Venue Labs, Bedford, OH). Six weeks after transplantation or control injection, animals were killed under pentobarbital anesthesia by aortic perfusion with 4% paraformaldehyde in 0.1 M PB, pH 7.4. The transplant-containing length of spinal cord was cut into 1-mm transverse blocks and processed so as to preserve the craniocaudal sequence and orientation. Even numbered tissue blocks were postfixed in 4% glutaraldehyde in 0.1 M PB, pH 7.4 for 3 days, rinsed in 0.1 M phosphate buffer, pH 7.4, for 30 min, postfixed in 1% OsO_4, dehydrated in ascending alcohols, and embedded in Spurr's resin. Blocks were trimmed and sections were cut at 100 nm, mounted on copper grids, uranyl acetate and lead citrate stained, and viewed under a Hitachi EM 600 electron microscope at 75 kV.

Immunohistochemical Staining

Odd-numbered tissue blocks were cryostat sectioned at 50 μm in the transverse or longitudinal plane and placed onto slides. Some sections were exposed to Methyl Green H-3402 (Vector) at 75°C for 8 min to label nuclei. Primary antisera (rat anti-BrdU, 1:200, Sigma-Aldrich; mouse anti-APC, CC-1 clone, 1:200, Oncogene Research Products, San Diego, CA; mouse anti-MBP, 1:10,000, Sternberger Monoclonals, Lutherville, MD) were visualized using either goat anti-mouse or rabbit anti-rat fluorescent antisera (Molecular Probes, Eugene, OR) or rabbit anti mouse biotinylated antisera (1:200, Vector Laboratories, Burlingame, CA) reacted with ABC solution (Vector Laboratories, Burlingame, CA) and DAB substrate solution (Vector). Photomicrographs were taken using an Olympus AX-80 light microscope.

RESULTS

Directed differentiation of oligodendrocytes and their progenitors from hESCs were maintained and amplified as previously described, in the absence of a feeder

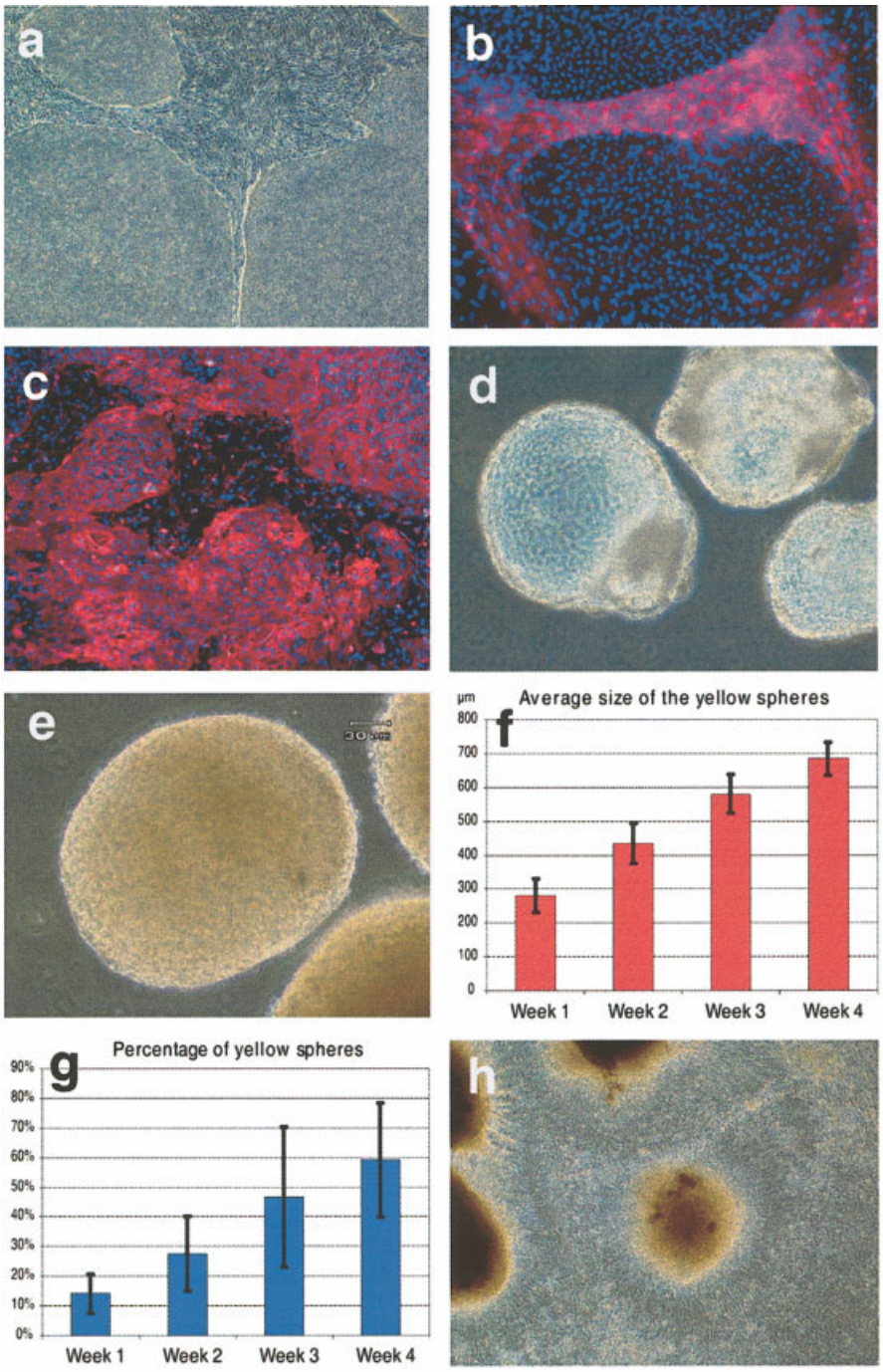

Fig. 1. Amplification and early differentiation of human embryonic stem cells (hESCs). **A:** Undifferentiated hESCs readily expanded, reaching confluence within 1 week of plating. **B:** hESCs grew as islands of cells separated by stromal cells immunoreactive for the mesodermal marker BMP4. **C:** hESC colonies expressed stage-specific embryonic antigen 4 (SSEA4). **D:** Transparent spheres appeared within 1 day of exposure to retinoic acid (RA) and increased in size and density during RA exposure, but were gradually eliminated after transferring cultures to RA-free GRM, indicating that they were not the source of oligodendroglial-lineage cells within the cultures. **E:** Yellow spheres appeared within 5 days of exposure to RA and grew rapidly in the presence of GRM, evidenced by an increase in their size (**F**) and proportion relative to other culture components (**G**). **H:** Plating yellow spheres on an adherent substrate resulted in the migration of cells from the sphere. Error bars illustrate standard deviation. A,H, ×40; B, ×200; C–E, ×100.

layer (Xu et al., 2001). In these conditions, hESC colonies (Fig. 1a) were observed within 1 day of cell passage, and confluence was attained within 7 days of passaging. hESC colonies were separated by stromal cells immunoreactive for the mesodermal marker BMP4 (Fig. 1b), which derived from the hESCs. hESC colonies expressed stage-specific embryonic antigen 4 (SSEA4; Fig. 1c), a surface marker for undifferentiated hESCs (Thomson et al., 1998).

Transfer of hESC cultures to 100% GRM resulted in extensive cell death (>90% of cells in culture). These findings led to our use of transition media (50% hESC growth media plus 50% GRM) for the first 2 days of differentiation, which resulted in >75% cell survival. Cultures were then exposed to 100% GRM for the duration of the differentiation protocol. In contrast to previous studies using mouse embryonic stem cells, we eliminated serum from all media formulations to prevent cellular maturation and differentiation (Barres et al., 1994). Although growth of cultures was equally robust when grown in GRM with 10% fetal bovine serum, or in GRM without insulin or selenium, these

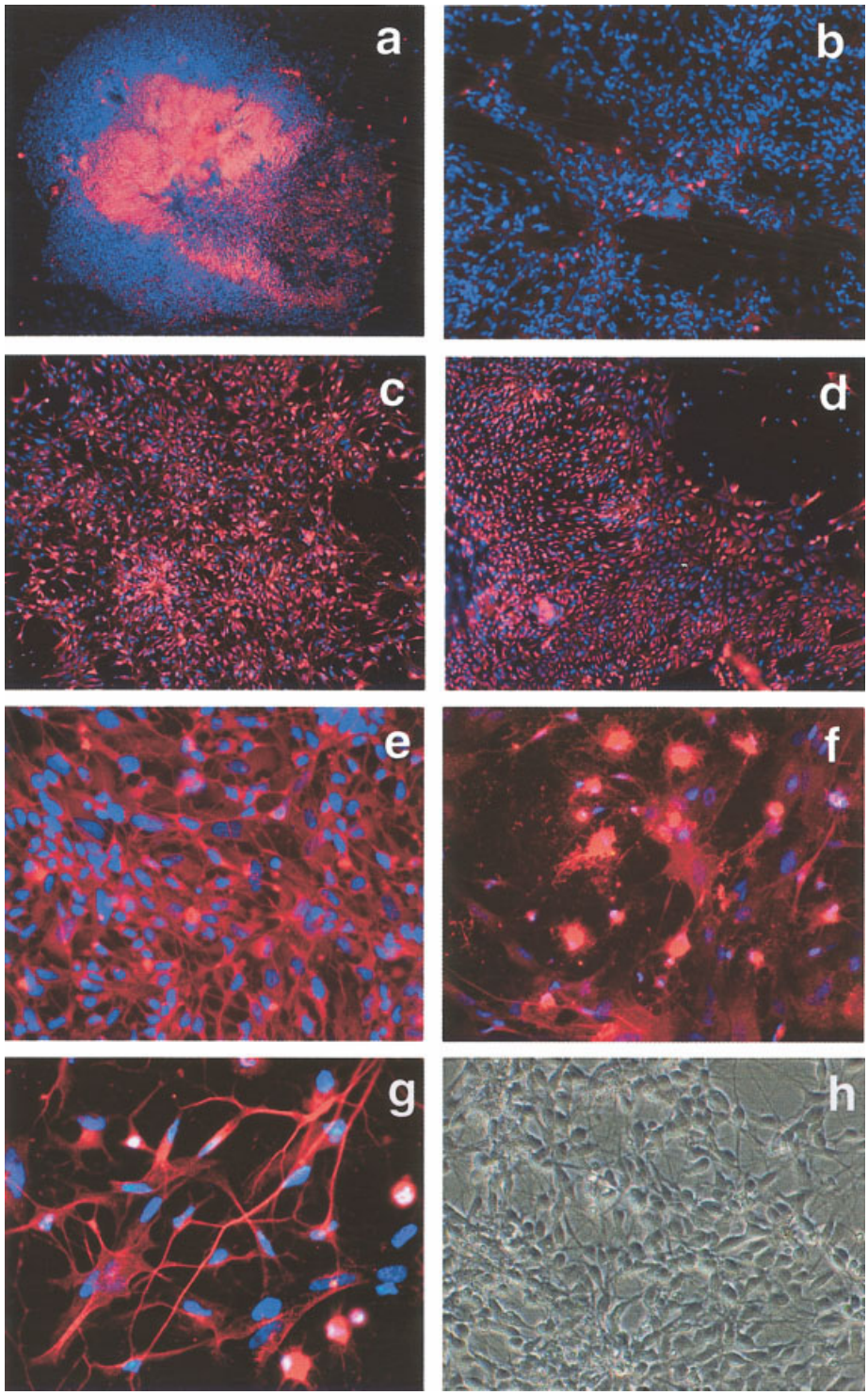

Fig. 2. Commitment of human embryonic stem cells (hESCs) to oligodendrocyte progenitors. Culture conditions preferentially promoted the growth of yellow spheres, which by day 10 of the 42-day differentiation protocol consisted of 98% ± 2% Pax6-positive cells within their central regions, and exceedingly few Pax6-positive cells within the periphery of spheres and amongst the migrating cells immediately adjacent to the spheres, indicating a neurogenic core (**A**). **B:** Pax6 expression was present in <1% of cells at the end of the differentiation protocol. **C:** 83% ± 7% of cells expressed the transcription factor Olig1 associated with oligodendrocyte and motor neuron specification. **D:** 72% ± 12% of cells expressed the DNA-binding protein SOX10 expressed within oligodendrocyte precursors. **E:** 97% ± 3% of cells expressed A2B5, a marker for oligodendrocyte progenitors. **F:** 98% ± 2% of cells expressed NG2, a marker for oligodendrocyte progenitors. **G:** 75% ± 20% of cells expressed PDGFRα, a marker for oligodendrocyte progenitors. **H:** At this stage of development, most cells displayed a bipolar morphology, characteristic of immature cells. A, ×40; B–D, ×100; E, H, ×400; F,G, ×600.

growth conditions yielded fewer oligodendroglial cells when carried through the rest of the differentiation protocol (results not shown).

The exposure of cultures to GRM at this early time point resulted in the emergence of transparent spheres of cells (Fig. 1d) and yellow spheres of cells (Fig. 1e). Transparent spheres began to appear within 1 day of exposure to RA. These transparent spheres increased in size and density during RA exposure, and were gradually eliminated after transferring cultures to RA-free GRM, indicating that they were not the source of glial cells within our cultures. Yellow spheres began to appear within 5 days of exposure to RA. GRM exposure promoted the preferential growth of yellow spheres within cultures, evidenced by an increase in the size (Fig. 1f) and proportion (Fig. 1g) of yellow spheres

during the differentiation protocol. The emergence of yellow spheres was decreased by >90% in cultures exposed to GRM with 10% fetal bovine serum relative to those exposed to GRM without serum.

The composition of yellow spheres at day 10 of the 42-day differentiation protocol was determined by plating individual yellow spheres on an adherent substrate at day 7 (Fig. 1h), and performing immunohistochemical analyses 3 days later (i.e., 1 day after removal of RA from the media). Plated yellow spheres contained 98% ± 2% Pax6-positive cells within the central region of the spheres, and exceedingly few Pax6-positive cells within the periphery of spheres and among the migrating cells immediately adjacent to the spheres (Fig. 2a). Pax6 expression was present in <1% of cells on day 42 of the differentiation protocol (Fig. 2b). As Pax6 is a potent neurogenic gene (Heins et al., 2002), these data indicate that most undifferentiated cells at early stages of the differentiation protocol are neural progenitors, and that more mature cultures contain few neural progenitors or neurons. That these neural progenitors adopt an oligodendroglial fate is indicated by the expression in cells within the periphery of spheres and immediately adjacent to the spheres of Olig1 (83% ± 7% of cells; Fig. 2c), SOX10 (72% ± 12% of cells; Fig. 2d), A2B5 (97% ± 3%; Fig. 2e), NG2 (98% ± 2%; Fig. 2f), and PDGFRα (75% ± 20%; Fig. 2g), as well as their bipolar morphology, characteristic of immature cells (Fig. 2h). No SSEA4-positive cells could be detected at this point in the differentiation protocol, indicating that these cultures were devoid of stem cells.

The content of oligodendroglial-lineage cells within cultures was enhanced by a mechanical enrichment step to select for yellow spheres. Yellow spheres were isolated on day 28 of the 42-day differentiation protocol by subjecting cultures to a mechanical selection procedure based upon preferential adhesion. Following transfer of free-floating cultures to adherent substrate overnight, yellow spheres preferentially bound to the substrate and were not dislodged by a gentle shaking and rinse. This procedure resulted in cultures that consisted of 100% adherent yellow spheres for the duration of the differentiation protocol.

After plating spheres at day 28 of the 42-day differentiation protocol, cells migrated progressively outward from the spheres and were maintained in an undifferentiated state by high-density growth in the presence of EGF. Cells displayed an immature morphology and continued to divide for up to 8 weeks following the end of the 42-day differentiation protocol (the latest time point analyzed). A 1:2 passaging was required weekly.

Cells were characterized in vitro at day 42 of the 42 day differentiation protocol or after amplification for 3, 6, or 8 additional weeks. Removal of EGF from the media and transfer of cells to a poly-L-lysine and human laminin substrate at low density (10,000 cells/cm^2) initiated the terminal differentiation of cells. Within 1 week, cells with a characteristic oligodendroglial morphology were evident (Fig. 3a–f), that labeled with GalC (Fig. 3g,h), a marker of oligodendroglia. Quantification of immunostained cultures indicated that 95% ± 4% of cells labeled with the oligodendroglial marker GalC (Fig. 4a), 95% ± 2% of cells labeled with the oligodendroglial marker RIP (Fig. 4b), and 85% ± 5% of cells labeled with the oligodendroglial marker 04 (Fig. 4c). Cells that did not label with oligodendroglial markers were primarily GFAP positive (Fig. 4d) or Tuj1 positive (Fig. 4e). Cultures demonstrated no immunoreactivity in no-primary-antibody immunocytochemical control staining chambers. Double immunohistochemical analyses indicated that no GFAP- or Tuj1-positive cells co-expressed oligodendroglial markers.

Furthermore, no BMP4- or SSEA4-positive cells could be detected, indicating that these cultures were devoid of mesodermal lineage cells or stem cells. This study was successfully conducted 8 times using cells from 8 subsequent passages.

Transplantation into the *shiverer* Mouse Model of Dysmyelination

Six weeks after transplantation of BrdU-pre-labeled cells from day 42 of our differentiation protocol, BrdU immunohistochemical analysis demonstrated that the transplanted cells survived and integrated almost exclusively within white matter (Fig. 5a). Double immunostaining for BrdU and CC-1 (Fig. 5b) indicated that transplanted $BrdU^+$ cells were $CC\text{-}1^+$ (Fig. 5c), indicating that they differentiated en masse to mature oligodendrocytes in vivo. BrdU and CC-1 double immunostained cells were distributed in patches throughout the white matter (Fig. 5a–c) up to 5 mm cranial and 5 mm caudal to the point of implantation (the furthest points examined). Electron microscopic analysis indicated that the 8 nontransplanted *shiverer* mice did not contain multilayered compact myelin (Fig. 5d), whereas all 8 transplanted *shiverer* mice contained multilayered compact myelin (Fig. 5e,f). In transplanted animals, multilayered compact myelin identified by electron microscopy appeared in patches throughout the white matter, up to 6 mm cranial and 6 mm caudal to the point of implantation (the farthest points examined). MBP immunostaining independently confirmed myelination by transplanted cells (Fig. 5g–i), revealing MBP^+ patches throughout the white matter, up to 5 mm cranial and 5 mm caudal to the point of implantation (the furthest points examined; BrdU, CC1 and MBP staining was conduced on odd numbered tissue blocks, whereas electron microscopy was conducted on even-numbered tissue blocks). The average number of MBP^+ patches per section was 18 (range 15–23) at the site of implantation, 8 (range 5–12) 5 mm cranial to the site of implantation, and 11 (range 9–14) 5 mm caudal to the site of implantation. Transplanted *shiverer* mice demonstrated no MBP, BrdU, or CC-1 reactivity on no-primary-antibody immunohistochemical control tissue sections. Nontrans-

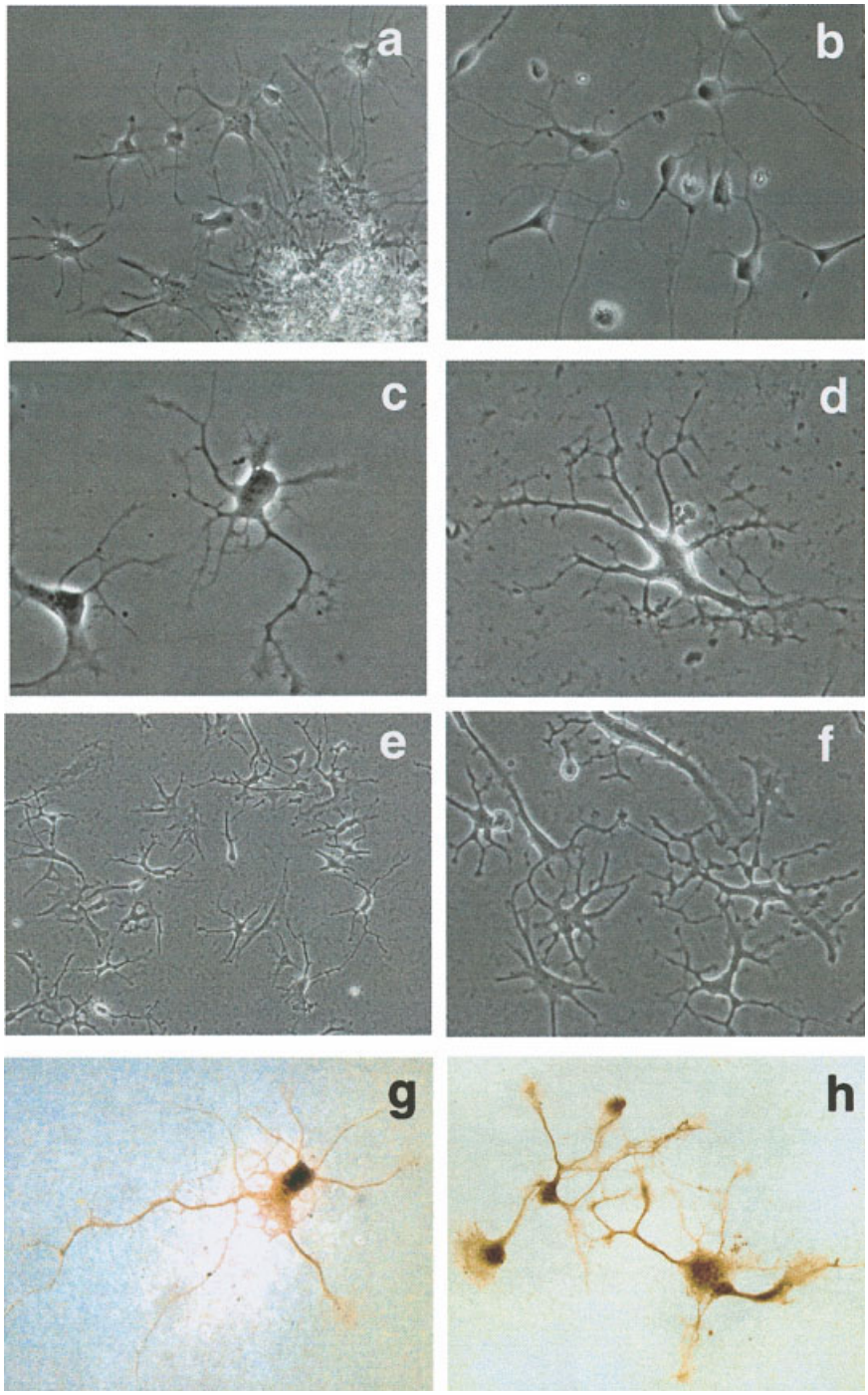

Fig. 3. Terminal differentiation of human embryonic stem cell (hESC)-derived oligodendrocytes evidenced by morphology in phase contrast, 1 week after plating at low density in the absence of growth factors. **A:** As cells migrate outward from plated spheres (bottom right-hand corner), they adopt the highly branched morphology typical of oligodendroglial cells. **B–F:** Plated cells adopted a typical oligodendroglial morphology, characterized by multiple branches with membranous extensions, and were GalC-positive (**G,H**). A,B,E, ×200; C,D,G,H, ×600; F,×100.

planted shiverer mice did not contain BrdU labeling, MBP labeling or compact myelin.

DISCUSSION

Oligodendrocytes are compromised in a number of neuropathological diseases including multiple sclerosis (Rodriguez et al., 1993), Pelizaeus-Merzbacher (Gow and Lazzarini, 1996), Alzheimer 's disease (Bartzokis, 2004), progressive multifocal leukoencephalopathy (Major et al., 1992), multisystem degeneration (Burn and Jaros, 2001), and oligodendrogliomas (Engelhard et al., 2003). The ability to generate human oligodendroglia en masse will allow studies of the biochemical and molecular mechanisms of oligodendrogliogenesis, studies that will have implications for the pathogenesis of these diseases, and also provide a means to generate donor cells for transplantation therapies. Research in rodent oligodendrocyte development over the past

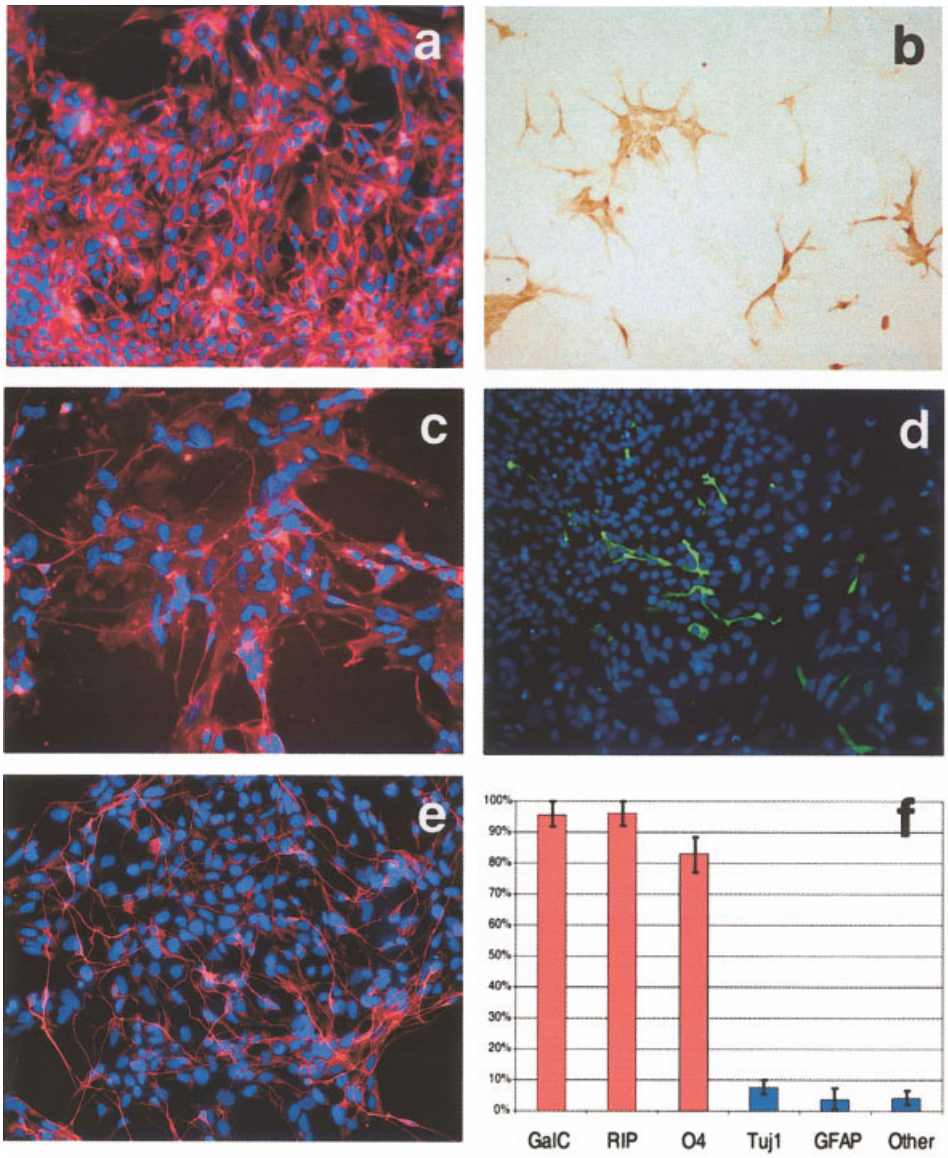

Fig. 4. Terminal differentiation of human embryonic stem cell (hESC)-derived oligodendrocytes evidenced by immunocytochemical staining, 1 week after plating at low density in the absence of growth factors. Greater than 95% of cells labeled with the oligodendroglial markers GalC (**A**), RIP (**B**), and 04 (**C**). Cells that did not label with oligodendroglial markers were primarily GFAP positive (**D**) or Tuj1 positive (**E**). **F:** Quantitation of immunolabeling. Error bars illustrate standard deviation. A,E, ×200; B,×400; C,D, ×100.

20 years has characterized the different phenotypic stages of the lineage, and identified many extrinsic signaling molecules that regulate developmental processes such as proliferation, migration, differentiation, and survival (see Grinspan, 2002). Benefiting from these studies, we have designed a protocol for the directed differentiation of oligodendrocytes, and applied it to hESCs. Here we present a method for the directed differentiation of human oligodendrocytes and their progenitors in high yield from federally approved hESCs, and demonstrate their functionality by illustrating compact MBP^+ myelin formation following transplantation into the *shiverer* model of dysmyelination.

Our differentiation protocol consisted of an induction of neural-lineage cells concurrent with the preferential selection of oligodendroglial-lineage cells by media components. We induced the formation of neural-lineage cells by exposing cultures to the classic RA caudalizing protocol (Bain et al., 1995; Wichterle et al., 2002) with several modifications. The RA protocol has traditionally involved a $4^-/4^+$ RA exposure during culture in an embryonic stem cell growth media (Carpenter et al., 2001). This protocol has been shown to reliably restrict differentiation to a multipotent neural lineage, which can generate neurons, oligodendrocytes, and astrocytes under appropriate conditions (Bain et al., 1995; Wichterle et al., 2002). With the goal of restricting the multipotent nature of this lineage to oligodendroglial-lineage cells, we exposed cultures to a novel glial restriction media (GRM) for 2 days prior to, and throughout an extended (7-day) RA exposure. The replacement of embryonic stem cell growth media with GRM at this early stage in the differentiation protocol resulted in death of most stem cells (>90%), necessitating the use of a transition media comprised of 50%

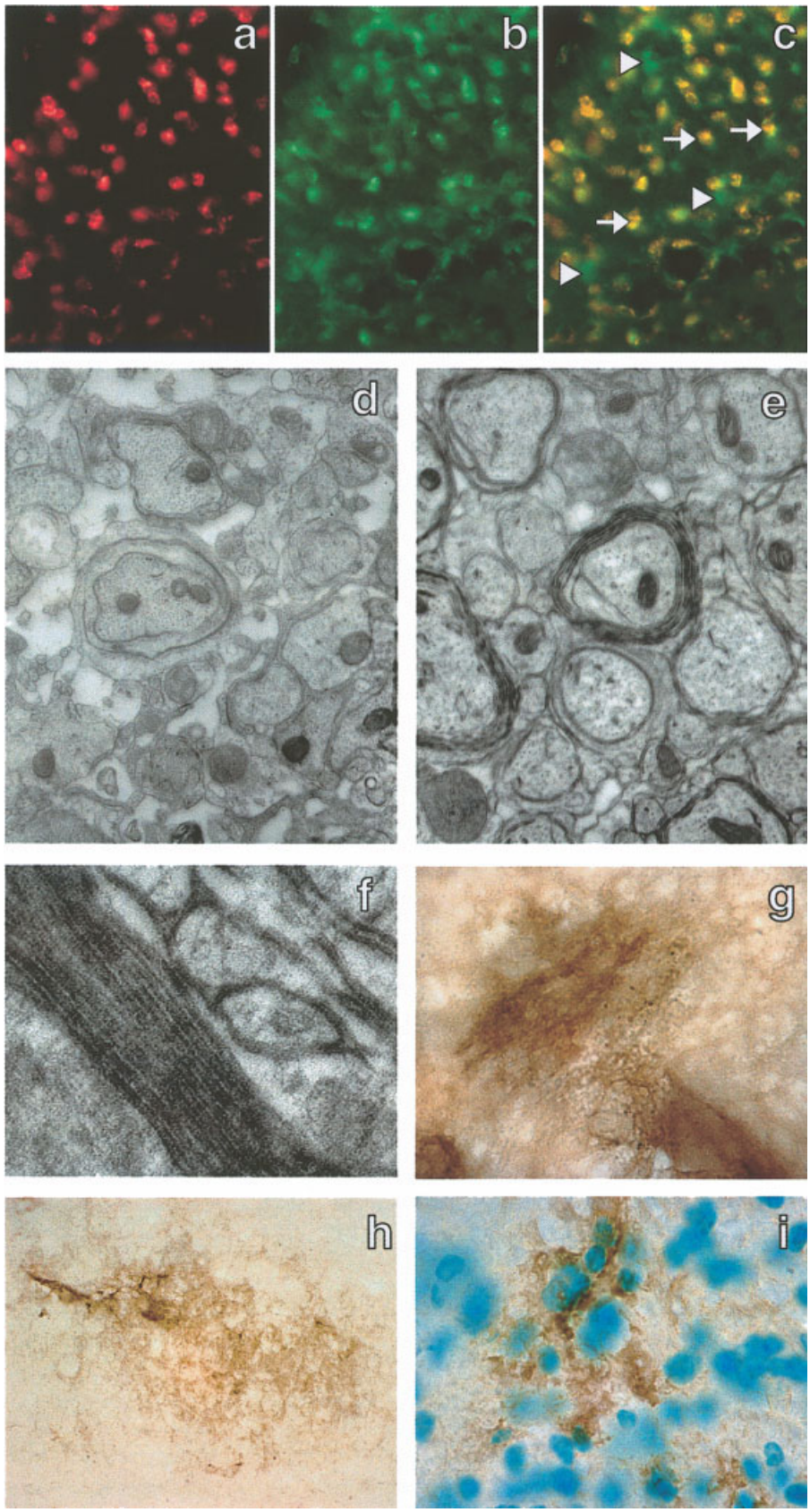

Fig. 5. Human embryonic stem cell (hESC)-derived oligodendrocytes integrate, differentiate and display a functional myelinating phenotype following transplantation into the *shiverer* mutant mouse. **A:** BrdU immunostaining illustrating the presence of transplanted BrdU pre-labeled cells within the spinal cord white matter. BrdU pre-labeled cells were found almost exclusively within spinal cord white matter. **B:** CC-1 immunostaining on the same section as (A) illustrating oligodendrocytes. **C:** Composite of BrdU and CC-1 double immunostaining, illustrating that transplanted $BrdU^+$ cells adopted the oligodendroglial marker CC-1. Arrows point to double labeled cells, arrowheads point to BrdU-$CC\text{-}1^+$ cells (endogenous *shiverer* oligodendrocytes). These panels indicate that transplanted BrdU pre-labeled cells survived and integrated within the spinal cord white matter, and became oligodendrocytes. **D:** Electron micrograph illustrating that axons of *shiverer* mice are devoid of myelin or are surrounded by one or two uncompacted wraps of myelin. **E:** Electron micrograph of the dorsal column white matter of a *shiverer* mouse 6 weeks after transplantation of hESC-derived oligodendrocyte progenitors, illustrating multilayered compact myelin. **F:** High-magnification image of compact myelin 6 weeks after transplantation of hESC-derived oligodendrocyte progenitors, illustrating multilayered compact myelin. *Shiverer* mice do not contain multilayered compact myelin. **E,F:** Myelination by transplanted cells. **G,H:** MBP immunopositive myelin patches within the dorsal column white matter of *shiverer* mice 6 weeks after transplantation of hESC-derived oligodendrocyte progenitors. **I:** MBP immunopositive myelin patches within the ventral column white matter of *shiverer* mice 6 weeks after transplantation of hESC-derived oligodendrocyte progenitors; nuclei are in blue. As *shiverer* mice do not produce MBP, G–I demonstrate myelination by transplanted cells. A,B,C,G,H, ×400; D,E, ×20,500; F, ×40,000; I, ×2,000.

embryonic stem cell growth media and 50% GRM, which resulted in survival of >75% of cells. These findings suggest that hESC cultures cannot tolerate a sudden and significant alteration of media components.

The principal GRM media components influencing oligodendroglial-lineage differentiation were the growth factor insulin (Barres et al., 1992), the differentiation factor triiodothyroidin hormone (Barres et al., 1994; Zhang and Lazar, 2000), and the growth factors FGF and EGF (Ben-Hur et al., 1998; Nakagaito et al., 1998). Insulin and insulin-like growth factors are regulators of oligodendrocyte development, and receptors for both substances are present on oligodendrocyte progenitors (Baron-Van Evercooren et al., 1991). In vitro, IGF-1 increases proliferation of oligodendrocyte precursors as well as the percentage of mature oligodendrocytes, likely as a result of the ability of IGF-1 to inhibit programmed cell death (McMorris and Dubois-Dalcq, 1988; Barres et al., 1992; Yasuda et al., 1995). Interestingly, IGF-1 overexpressing transgenic mice have significantly more oligodendrocytes and myelin per oligodendrocyte than wild-type controls (Carson et al., 1993), suggesting that IGF-1 plays a critical role in myelination and oligodendrocyte survival. Thyroid hormone affects the oligodendrocyte lineage at multiple points in its development. Thyroid hormone plays a role in proliferation and survival of oligodendrocyte pre-progenitors (Ben-Hur et al., 1998), while it promotes differentiation (Ibarrola et al., 1996) and regulates timing of differentiation (Barres et al., 1994) in oligodendrocytes. Both thyroid hormone-deficient transgenic mice and hypothyroid rodents contain substantially decreased oligodendrocytes, demonstrating a critical role for thyroid hormone in oligodendrogliogenesis. The growth factors FGF and EGF were employed based on their ability to stimulate extended proliferation of neural progenitor cells and promote glial cell differentiation, particularly of oligodendrocytes (Ben-Hur et al., 1998; Benoit et al., 2001). Despite the demonstrated role of these factors in oligodendrogliogenesis, spontaneous differentiation of hESCs may have contributed to our total oligodendrocyte lineage yield. The ability of hESCs to differentiate spontaneously has been well documented (Itskovitz-Eldor et al., 2000; Reubinoff et al., 2000; Schuldiner et al., 2000; Levenberg et al., 2002; Xu et al., 2002).

Oligodendroglial lineage commitment was confirmed at day 10 of the 42-day differentiation protocol, using a panel of markers that included Olig1, SOX10, A2B5, NG2, and PDGFRα (Fig. 2). It is important to note that, while one marker alone is insufficient to determine lineage, this panel of markers definitively demonstrates oligodendroglial lineage commitment. The necessity of a panel of markers for oligodendroglial identification underscores the need within the research community for oligodendroglial specific markers. A2B5, NG2, and PDGFRα are markers for oligodendrocyte progenitors (Abney et al., 1983; Raff et al., 1983; Levine and Stallcup, 1987; Stallcup and Beasley, 1987), although A2B5 and NG2 may stain other cell populations in human tissue as well (Rakic and Zecevic, 2003). The Olig1 and Olig2 transcription factors are critical for oligodendrocyte and motor neuron specification (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000), and SOX10, a member of the high-mobility group domain family of DNA binding proteins, is expressed within oligodendrocyte precursors, and is not expressed in neurons or astrocytes (Kuhlbrodt et al., 1998). Taken together, these data indicate that oligodendroglial lineage commitment was prominent by day 10 of the differentiation protocol.

Because of the relatively slow rate of growth of human cells and the high costs associated with human cell culture, we developed an amplification step at the end of the differentiation protocol. Cells were amplified in an undifferentiated state by high-density growth in the presence of EGF for up to 8 weeks (the latest time point analyzed). A 1:2 passaging was required weekly, indicating that this stage in the differentiation protocol represents a significant amplification step. This finding is particularly important for studies or therapies which require large numbers of cells, where mass culture of a hESC starting population is not practical or cost-effective.

The development of oligodendroglia was confirmed at the end of the differentiation protocol by their morphology and antigenic expression (Figs. 3 and 4). These data indicate that this differentiation protocol generated high-purity oligodendrocyte cultures. This study was successfully conducted 8 times using cells from eight subsequent passages, indicating that hESCs maintained in culture for extensive periods retain the potential to differentiate.

Having demonstrated oligodendrocyte progenitor (Fig. 2) and oligodendrocyte (Figs. 3 and 4) development in vitro, we then demonstrated their ability to integrate, differentiate and display a functional phenotype (produce myelin) in vivo by transplantation into the homozygous *shiverer* mouse model of dysmyelination (Fig. 5). *Shiverer* mice are homozygous for a mutation in the myelin basic protein (MBP) gene located on chromosome 18 (Mbp^{shi}/Mbp^{shi}), causing severe myelin deficiency throughout the CNS (Chernoff, 1981). Such mice do not produce MBP which is essential for myelin compaction, and contain axons that are devoid of myelin or are surrounded by one or two uncompacted wraps of myelin (Gansmuller et al., 1986; Yandava et al., 1999). Because *shiverer* mice do not contain compact myelin or MBP, detection of multilayered compact myelin and MBP immunostaining following transplantation into *shiverer* mice has been used as the gold standard for demonstrating myelination from transplanted oligodendrocytes (Gansmuller et al., 1986, 1991; Gumpel et al., 1987; Warrington et al., 1993; Seilhean et al., 1996; Yandava et al., 1999; Liu et al., 2000; Franklin, 2002). Our findings demonstrate the ability of transplanted cells to integrate, differentiate into oligodendrocytes and display a functional phenotype, thereby confirming oligodendrogliogenesis from hESCs.

This study demonstrates for the first time that hESCs can be directed in their differentiation to generate high-purity oligodendroglial lineage cultures that display a functional myelinating phenotype following transplantation. The availability of hESCs (Thomson et al., 1998; Carpenter et al., 2001), the ability to direct their differentiation to the oligodendroglial lineage, and the possibility of engineering autologous hESCs by somatic nuclear transfer, raise exciting possibilities for the treatment of several human diseases and injury states involving demyelination. Furthermore, they provide important tools for investigating the mechanisms controlling human oligodendrocyte differentiation and myelination.

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REFERENCES

Abney ER, Williams BP, Raff MC. 1983. Tracing the development of oligodendrocytes from precursor cells using monoclonal antibodies, fluorescence-activated cell sorting, and cell culture. Dev Biol 100: 166–171.

Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. 1995. Embryonic stem cells express neuronal properties in vitro. Dev Biol 168: 342–357.

Baron-Van Evercooren A, Olichon-Berthe C, Kowalski A, Visciano G, Van Obberghen E. 1991. Expression of IGF-I and insulin receptor genes in the rat central nervous system: a developmental, regional, and cellular analysis. J Neurosci Res 28:244–253.

Barres BA, Hart IK, Coles HS, Burne JF, Voyvodic JT, Richardson WD, Raff MC. 1992. Cell death and control of cell survival in the oligodendrocyte lineage. Cell 70:31–46.

Barres BA, Lazar MA, Raff MC. 1994. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120:1097–1108.

Bartzokis G. 2004. Age-related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. Neurobiol Aging 25:5–18; author reply 49–62.

Ben-Hur T, Rogister B, Murray K, Rougon G, Dubois-Dalcq M. 1998. Growth and fate of PSA-NCAM$^+$ precursors of the postnatal brain. J Neurosci 18:5777–5788.

Benoit BO, Savarese T, Joly M, Engstrom CM, Pang L, Reilly J, Recht LD, Ross AH, Quesenberry PJ. 2001. Neurotrophin channeling of neural progenitor cell differentiation. J Neurobiol 46:265–280.

Brustle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD. 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285: 754–756.

Burn DJ, Jaros E. 2001. Multiple system atrophy: cellular and molecular pathology. Mol Pathol 54:419–426.

Carpenter MK, Inokuma MS, Denham J, Mujtaba T, Chiu CP, Rao MS. 2001. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 172:383–397.

Carson MJ, Behringer RR, Brinster RL, McMorris FA. 1993. Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron 10:729–740.

Chernoff GF. 1981. Shiverer: an autosomal recessive mutant mouse with myelin deficiency. J Hered 72:128.

Deacon T, Dinsmore J, Costantini LC, Ratliff J, Isacson O. 1998. Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. Exp Neurol 149:28–41.

Engelhard HH, Stelea A, Mundt A. 2003. Oligodendroglioma and anaplastic oligodendroglioma: clinical features, treatment, and prognosis. Surg Neurol 60:443–456.

Franklin RJ. 2002. Remyelination of the demyelinated CNS: the case for and against transplantation of central, peripheral and olfactory glia. Brain Res Bull 57:827–832.

Gansmuller A, Lachapelle F, Baron-Van Evercooren A, Hauw JJ, Baumann N, Gumpel M. 1986. Transplantations of newborn CNS fragments into the brain of shiverer mutant mice: extensive myelination by transplanted oligodendrocytes. II. Electron microscopic study. Dev Neurosci 8:197–207.

Gansmuller A, Clerin E, Kruger F, Gumpel M, Lachapelle F. 1991. Tracing transplanted oligodendrocytes during migration and maturation in the shiverer mouse brain. Glia 4:580–590.

Gow A, Lazzarini RA. 1996. A cellular mechanism governing the severity of Pelizaeus-Merzbacher disease. Nat Genet 13:422–428.

Grinspan J. 2002. Cells and signaling in oligodendrocyte development. J Neuropathol Exp Neurol 61:297–306.

Gumpel M, Lachapelle F, Gansmuller A, Baulac M, Baron van Evercooren A, Baumann N. 1987. Transplantation of human embryonic oligodendrocytes into shiverer brain. Ann NY Acad Sci 495:71–85.

Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde YA, Gotz M. 2002. Glial cells generate neurons: the role of the transcription factor Pax6. Nat Neurosci 5:308–315.

Ibarrola N, Mayer-Proschel M, Rodriguez-Pena A, Noble M. 1996. Evidence for the existence of at least two timing mechanisms that contribute to oligodendrocyte generation in vitro. Dev Biol 180:1–21.

Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N. 2000. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol Med 6:88–95.

Keirstead HS. 2001. Stem cell transplantation into the central nervous system and the control of differentiation. J Neurosci Res 63:233–236.

Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 418:50–56.

Kim JY, Koh HC, Lee JY, Chang MY, Kim YC, Chung HY, Son H, Lee YS, Studer L, McKay R, Lee SH. 2003. Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. J Neurochem 85:1443–1454.

Klug MG, Soonpaa MH, Koh GY, Field LJ. 1996. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. J Clin Invest 98:216–224.

Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M. 1998. Sox10, a novel transcriptional modulator in glial cells. J Neurosci 18:237–250.

Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. 2000. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 18:675–679.

Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. 2002. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 99:4391–4396.

Levine JM, Stallcup WB. 1987. Plasticity of developing cerebellar cells in vitro studied with antibodies against the NG2 antigen. J Neurosci 7:2721–2731.

Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF, McDonald JW. 2000. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci USA 97:6126–6131.

Loebel DA, Watson CM, De Young RA, Tam PP. 2003. Lineage choice and differentiation in mouse embryos and embryonic stem cells. Dev Biol 264:1–14.

Lu QR, Yuk D, Alberta JA, Zhu Z, Pawlitzky I, Chan J, McMahon AP, Stiles CD, Rowitch DH. 2000. Sonic hedgehog-regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. Neuron 25:317–329.

Major EO, Amemiya K, Tornatore CS, Houff SA, Berger JR. 1992. Pathogenesis and molecular biology of progressive multifocal leukoencephalopathy, the JC virus-induced demyelinating disease of the human brain. Clin Microbiol Rev 5:49–73.

McMorris FA, Dubois-Dalcq M. 1988. Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. J Neurosci Res 21:199–209.

Nakagaito Y, Satoh M, Kuno H, Iwama T, Takeuchi M, Hakura A, Yoshida T. 1998. Establishment of an epidermal growth factor-dependent, multipotent neural precursor cell line. In Vitro Cell Dev Biol Anim 34:585–592.

Nishimura F, Yoshikawa M, Kanda S, Nonaka M, Yokota H, Shiroi A, Nakase H, Hirabayashi H, Ouji Y, Birumachi J, Ishizaka S, Sakaki T. 2003. Potential use of embryonic stem cells for the treatment of

mouse parkinsonian models: improved behavior by transplantation of in vitro differentiated dopaminergic neurons from embryonic stem cells. Stem Cells 21:171–180.
Pera MF, Andrade J, Houssami S, Reubinoff B, Trounson A, Stanley EG, Oostwaard DW, Mummery C. 2004. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. J Cell Sci 117:1269–1280.
Raff MC, Miller RH, Noble M. 1983. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. Nature 303:390–396.
Rakic S, Zecevic N. 2003. Early oligodendrocyte progenitor cells in the human fetal telencephalon. Glia 41:117–127.
Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. 2000. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18:399–404.
Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T. 2001. Neural progenitors from human embryonic stem cells. Nat Biotechnol 19:1134–1140.
Rodriguez M, Scheithauer BW, Forbes G, Kelly PJ. 1993. Oligodendrocyte injury is an early event in lesions of multiple sclerosis. Mayo Clin Proc 68:627–636.
Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 97:11307–11312.
Seilhean D, Gansmuller A, Baron-Van Evercooren A, Gumpel M, Lachapelle F. 1996. Myelination by transplanted human and mouse central nervous system tissue after long-term cryopreservation. Acta Neuropathol (Berl) 91:82–88.
Soria B, Roche E, Berna G, Leon-Quinto T, Reig JA, Martin F. 2000. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes 49:157–162.
Stallcup WB, Beasley L. 1987. Bipotential glial precursor cells of the optic nerve express the NG2 proteoglycan. J Neurosci 7:2737–2744.
Takebayashi H, Yoshida S, Sugimori M, Kosako H, Kominami R, Nakafuku M, Nabeshima Y. 2000. Dynamic expression of basic helix-loop-helix Olig family members: implication of Olig2 in neuron and oligodendrocyte differentiation and identification of a new member, Olig3. Mech Dev 99:143–148.
Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.
Warrington AE, Barbarese E, Pfeiffer SE. 1993. Differential myelinogenic capacity of specific developmental stages of the oligodendrocyte lineage upon transplantation into hypomyelinating hosts. J Neurosci Res 34:1–13.
Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. Cell 110: 385–397.
Wiles MV, Keller G. 1991. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111:259–267.
Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. 2001. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971–974.
Xu C, Police S, Rao N, Carpenter MK. 2002. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 91:501–508.
Yandava BD, Billinghurst LL, Snyder EY. 1999. "Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. Proc Natl Acad Sci USA 96:7029–7034.
Yasuda T, Grinspan J, Stern J, Franceschini B, Bannerman P, Pleasure D. 1995. Apoptosis occurs in the oligodendroglial lineage, and is prevented by basic fibroblast growth factor. J Neurosci Res 40: 306–317.
Zhang J, Lazar MA. 2000. The mechanism of action of thyroid hormones. Annu Rev Physiol 62:439–466.
Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 19:1129–1133.
Zhou Q, Wang S, Anderson DJ. 2000. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron 25:331–343.