In vitro differentiation of transplantable neural precursors from human embryonic stem cells

Su-Chun Zhang[1,2,4]*, Marius Wernig[5], Ian D. Duncan[3], Oliver Brüstle[5]*, and James A. Thomson[1]

The remarkable developmental potential and replicative capacity of human embryonic stem (ES) cells promise an almost unlimited supply of specific cell types for transplantation therapies. Here we describe the *in vitro* differentiation, enrichment, and transplantation of neural precursor cells from human ES cells. Upon aggregation to embryoid bodies, differentiating ES cells formed large numbers of neural tube–like structures in the presence of fibroblast growth factor 2 (FGF-2). Neural precursors within these formations were isolated by selective enzymatic digestion and further purified on the basis of differential adhesion. Following withdrawal of FGF-2, they differentiated into neurons, astrocytes, and oligodendrocytes. After transplantation into the neonatal mouse brain, human ES cell–derived neural precursors were incorporated into a variety of brain regions, where they differentiated into both neurons and astrocytes. No teratoma formation was observed in the transplant recipients. These results depict human ES cells as a source of transplantable neural precursors for possible nervous system repair.

Human ES cells are pluripotent cells derived from the inner cell mass of preimplantation embryos[1]. Like mouse ES cells, they can be expanded to large numbers while maintaining their potential to differentiate into various somatic cell types of all three germ layers[1–4]. The *in vitro* differentiation of ES cells provides new perspectives for studying the cellular and molecular mechanisms of early development and the generation of donor cells for transplantation therapies. Indeed, mouse ES cells have been found to differentiate *in vitro* to many clinically relevant cell types, including hematopoietic cells[5], cardiomyocytes[6], insulin-secreting cells[7], neurons, and glia[8–12]. Following transplantation into the rodent central nervous system (CNS), ES cell–derived neural precursors have been shown to integrate into the host tissue[12] and, in some cases, yield functional improvement[13]. A clinical application of human ES cells would require the generation of highly purified donor cells for specific tissues and organs. Here we describe a simple yet efficient strategy for the isolation of transplantable neural precursors from differentiating human ES cell cultures.

Results

Human ES cells differentiate to form neural tube–like structures in the presence of FGF-2. Human ES cell lines H1, H9, and a clonal line derived from H9, H9.2 (ref. 4), were propagated on a feeder layer of irradiated mouse embryonic fibroblasts[1]. To initiate differentiation, ES cell colonies were detached and grown in suspension as embryoid bodies (EBs) for four days. The EBs were then cultured in a tissue culture–treated flask in a chemically defined medium[14,15] containing FGF-2. After five days of culture in FGF-2, the plated EBs had generated an outgrowth of flattened cells. At the same time, an increasing number of small, elongated cells were noted in the center of the differentiating EBs (Fig. 1A). By seven days in the defined medium, the central, small, elongated cells had generated rosette formations (Fig. 1B) resembling the early neural tube, as shown by toluidine blue–stained sections (inset in Fig. 1B). Immunofluorescence analyses revealed that the expression of the neural marker antigens nestin and Musashi-1 (refs 16, 17) was largely restricted to the cells in the rosettes but not the flat cells at the periphery of the differentiating EBs (Fig. 1C–E). The flat cells were immunonegative for several markers of differentiated neurons and glia: neurofilament 68, O4, O1, and glial fibrillary acidic protein (GFAP). They were also negative for alkaline phosphatase, whereas undifferentiated ES cells were positive as reported elsewhere[1]. Undifferentiated ES cells were negative for the neuroepithelial markers tested. The formation of neural tube–like structures was noted in the majority of EBs in the presence of FGF-2 (94% of the total 350 EBs from H9 and H9.2 lines, three separate experiments). In the absence of FGF-2, no well-organized rosettes were observed.

Neural tube–like rosettes can be isolated by differential enzymatic treatment and adhesion. With continued exposure to FGF-2, the columnar rosette cells expanded and formed multiple layers. They frequently made up most of the EB and were sharply demarcated from the surrounding flat cells. Treatment with dispase led to the preferential detachment of the central neuroepithelial islands, leaving the surrounding cells largely adherent (Fig. 1F). Contaminating single cells were separated by short-term adhesion to cell culture dishes. Cell counts performed immediately after this isolation and enrichment procedure showed that cells associated with the isolated neuroepithelial clusters represented 72–84% of the cells in the differentiated EB cultures. Immunocytochemical analyses showed that 96 ± 0.6% of the isolated rosette cells were positively stained for nestin, on the basis of 13,324 cells examined in four separate experiments. The vast majority of these cells were also positive for Musashi-1 and polysialylated neuronal cell adhesion molecule (PSA-NCAM) (Fig. 1G–I).

Human ES cell–derived neural precursors generate all three CNS cell types *in vitro*. The isolated neural precursors were expanded as free-floating cell aggregates in a suspension culture,

[1]*Departments of Anatomy,* [2]*Neurology, and* [3]*Medical Sciences, and* [4]*the Waisman Center, University of Wisconsin, 1500 Highland Avenue, Madison, WI 53705.*
[5]*Department of Neuropathology, University of Bonn Medical Center, Sigmund-Freud-Strasse 25, 53105 Bonn, Germany.*
**Corresponding authors (zhang@waisman.wisc.edu; brustle@uni-bonn.de).*

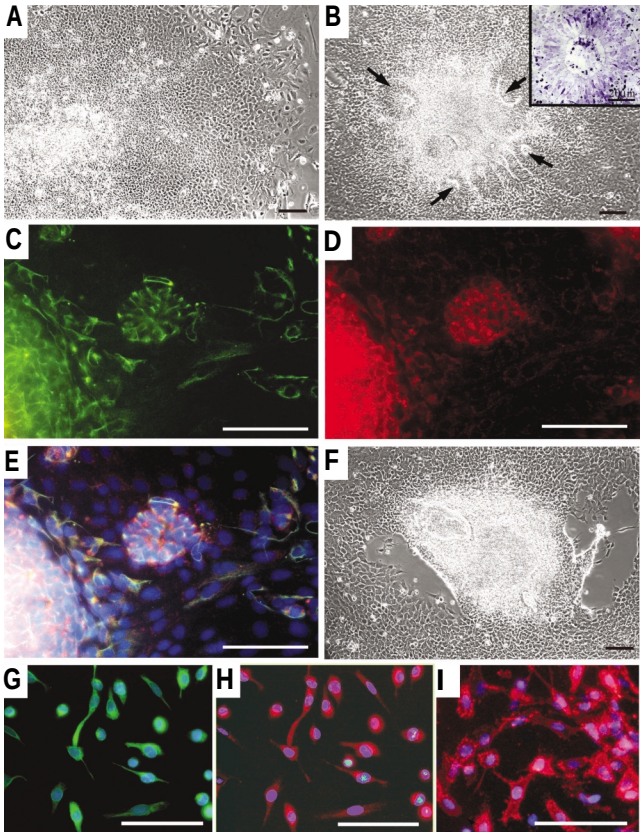

Figure 1. Differentiation and isolation of neural precursors from ES cells. (A) An attached EB grown in the presence of FGF-2 for five days shows flattened cells at the periphery and small elongated cells congregated in the center. (B) By seven days, many rosette formations (arrows) appear in the center of the differentiating EB. Inset: 1 μm section of the rosette formation stained with toluidine blue, showing columnar cells arranging in a tubular structure. Bar, 20 μm. (C–E) Cells within a cluster of rosettes (lower left) and a small evolving rosette (center) are positive for nestin (C) and Musashi-1 (D), while the surrounding flat cells are negative. (E) A combined image of (C) and (D) with all cell nuclei labeled with DAPI. (F) After treatment with dispase for 20 min, the rosette formations retracted, while the surrounding flat cells remained attached. (G–I) Isolated cells are positively stained for nestin in a filamentous pattern (G), Musashi-1 in cytoplasm (H), and PSA-NCAM mainly on membrane (I). All nuclei are stained with DAPI. Bars = 100 μm.

similar to "neurosphere" cultures derived from human fetal brain tissues[14,18–24]. Bromodeoxyuridine (BrdU) incorporation studies revealed that stimulation of precursor cell proliferation was dependent on FGF-2 and could not be elicited by either EGF or leukemia inhibitory factor (LIF) alone. Furthermore, no additive or synergistic effects were observed when FGF-2 was combined with EGF and/or LIF (Fig. 2A). ES cell–derived neurospheres split every other week and maintained up to eight passages differentiated into neurons and glia in a similar pattern as early passages (see below).

In vitro differentiation of the ES cell–derived neural precursors was induced by withdrawal of FGF-2 and plating on ornithine and laminin substrate. Within a few days, individual cells and numerous processes grew out from the spheres, giving a starburst appearance. By 7–10 days after plating, processes emanating from the spheres had formed prominent fiber bundles. Frequently, small migrating cells were seen in close association with the fibers (Fig. 2B). Immunofluorescence analyses of the differentiated cultures revealed that the vast majority of cells in the outgrowth areas expressed neuronal markers MAP2ab and β_{III}-tubulin (Fig. 2C). Expression of low- and high-molecular-weight neurofilament (NF) was observed by 7–10 and 10–14 days after plating, respectively (Fig. 2D). Antibodies to various neurotransmitters were used to further characterize the ES cell–derived neurons. While the majority of the neurons exhibited a glutamatergic phenotype (Fig. 2E), a smaller proportion was labeled with an antibody to γ-aminobutyric acid (GABA). Frequently, these neurons showed a polar morphology (Fig. 2F). A small number of neurons were found to express tyrosine hydroxylase (TH; Fig. 2G), the rate-limiting enzyme in dopamine synthesis. $GFAP^+$ astrocytes were rarely found within the first two weeks after growth factor withdrawal (Fig. 2C) but became more frequent after prolonged *in vitro* differentiation. By six to seven weeks, they had formed an extensive layer underneath the differentiated neurons (Fig. 2D). While oligodendrocytes were not observed under standard culture conditions, a few O4-immunoreactive cells with a typical multipolar oligodendroglial morphology were observed when the cells were cultured in the presence of platelet-derived growth factor A (PDGF-A; ref. 14) for longer than two weeks (Fig. 2H). Neural precursor cells derived from ES cell lines H1, H9, and H9.2 showed a similar pattern of neural differentiation. Thus, ES cell–derived neural precursors were able to generate all three major cell types of the CNS.

Human ES cell–derived neural precursors migrate, incorporate, and differentiate *in vivo*. To assess the differentiation of human ES cell–derived neural precursors *in vivo*, we grafted them into the lateral ventricles of newborn mice[21]. The transplanted cells formed clusters in various regions of the ventricular system and incorporated in large numbers into a variety of host brain regions. A slight enlargement of the ventricular system was noted in some of the transplant recipients. Of 22 brains analyzed between one and four weeks after transplantation, intraventricular clusters and incorporated cells were found in 19 and 18 recipient brains, respectively. Hence, the majority of the transplanted animals contained both clusters and incorporated cells. Individual animals analyzed after longer time periods showed that grafted cells were detectable for at least eight weeks post transplantation. The clusters were composed of densely packed and evenly distributed cells exhibiting immunoreactivity to antibodies against nestin, β_{III}-tubulin, and MAP2ab (Fig. 3). Only a few cells in the aggregates expressed GFAP. Intraventricular clusters and incorporated donor cells were negative for alkaline phosphatase and cytokeratin, markers typically expressed in undifferentiated ES cells and non-neural epithelia. No teratoma formation was observed.

DNA *in situ* hybridization with a human-specific probe and immunohistochemical detection of a human nucleus-specific antigen revealed the presence of grafted cells in numerous brain regions. Gray matter areas exhibiting widespread donor cell incorporation included cortex (Fig. 4A), hippocampus (Fig. 4B,C), olfactory bulb, septum (Fig. 4D), thalamus, hypothalamus (Fig. 4E), striatum (Fig. 4F), and midbrain (Fig. 4G). Four weeks after transplantation, a quantification of incorporated cells in three selected regions revealed densities of 35 (cortex), 24 (striatum), and 116 (tectum) cells per 50 μm section (mean number recruited from four animals, three sections per region).

Incorporation into white-matter regions was most pronounced in the corpus callosum, internal capsule, and hippocampal fiber tracts. Morphologically, the incorporated human cells were indistinguishable from the surrounding host cells and only detectable by the use of human-specific markers (Fig. 4). Double labeling with cell type–specific antibodies revealed that the incorporated cells had differentiated into both neurons and glia. Large numbers

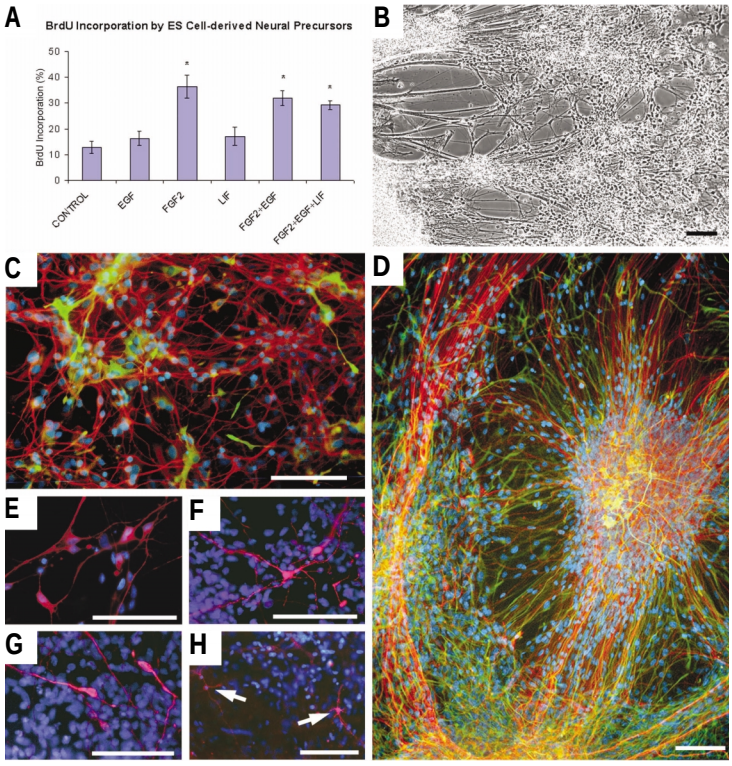

Figure 2. Characterization of ES cell–derived neural precursors *in vitro*. (A) BrdU incorporation by dissociated ES cell–derived neural precursors is enhanced in the presence of FGF-2 (20 ng/ml) but not EGF (20 ng/ml) or LIF (5 ng/ml). These are representative data from one of three replicate experiments. Asterisks indicate difference between the experimental group and the control group ($P < 0.01$, $n = 4$, Student's *t*-test). Neither EGF nor LIF alone affected the rate of BrdU incorporation. No synergistic effects were obtained by combining LIF with either EGF or FGF alone (not shown). (B) Differentiation of a cluster of ES cell–derived neural precursors for three weeks shows neurite bundles with cells migrating along them. (C) Immunostaining after three weeks of differentiation indicates that the majority of cells are β_{III}-tubulin$^+$ neurons (red) and that only a few cells are GFAP$^+$ astrocytes (green). (D) After 45 days of differentiation, many more GFAP$^+$ astrocytes (green) appear along with NF200$^+$ neurites (red, yellowish due to overlapping with green GFAP). (E–G) ES cell–derived neurons with various morphologies express distinct neurotransmitters such as glutamate (E), GABA (F), and the enzyme tyrosine hydroxylase (G). O4$^+$ oligodendrocytes (arrows) are observed after two weeks of differentiation in a glial differentiation medium (H). Bars = 100 μm.

of human ES cell–derived neurons could be clearly delineated with antibodies to β_{III}-tubulin and MAP2 (Fig. 4H, J). Frequently, they displayed uni- and bipolar morphologies with long processes (Fig. 4H). In addition, neurons with multipolar neurites were found (Fig. 4J). The donor-derived neurons generated numerous axons projecting long distances into the host brain, which were detected in both gray and white matter. They were particularly abundant within fiber tracts such as the corpus callosum, the anterior commissure, and the fimbria hippocampi, where they could frequently be traced for several hundred micrometers within a single section (Fig. 4I).

In addition to neurons, a small number of ES cell–derived astrocytes were detected within the host brain tissue. They displayed stellate morphologies and exhibited strong expression of GFAP (Fig. 4K). In contrast, double labeling of incorporated human cells with antibodies to myelin proteins failed to detect mature oligodendrocytes. Some of the donor cells that had migrated into the host brain retained a nestin-positive phenotype even up to four weeks after transplantation. Many of these cells were found in perivascular locations.

Discussion

The present study indicates that engraftable neural precursors capable of generating mature neurons and glia can be prepared with high yield from human ES cells. Exploiting growth factor treatment and differential adhesion of neural precursor cells, the *in vitro* differentiation procedure described here provides a platform for the study of neural development and for the generation of donor cells for possible nervous system repair.

An interesting finding of this study is the observation that the *in vitro* differentiation of neural precursors from human ES cells appears to recapitulate early steps of nervous system development in that neural tube–like structures are formed. Similar observations have been made following intraventricular transplantation of mouse ES cell–derived neural precursors into the embryonic rat brain[12]. In contrast to this previous study, our study found that human cells formed neural tube–like structures only *in vitro*. From a developmental perspective, this phenomenon could serve as an experimental tool to study human neural tube formation under controlled conditions.

On a pragmatic level, the *in vitro* generation of neural tube–like structures and the possibility of isolating these structures on the basis of their differential adhesion provides a simple yet efficient approach for generating human ES cell–derived neural precursors in high purity. Specifically, the strong cell–cell contacts within the neuroepithelial structures and their low adhesiveness to the tissue culture substrate permits the selective isolation of neural cells without significant contamination by cells of other somatic lineages. More than 95% of the isolated cells exhibited a nestin-positive phenotype, and no ES cells or non-neural epithelia were detectable in transplanted recipients. Because undifferentiated ES cells and precursors to other lineages may form tumors and foreign tissues, the generation of purified somatic cell populations is a key prerequisite for the development of ES cell–based neural transplant strategies.

Reubinoff and colleagues have previously reported *in vitro* differentiation and isolation of human ES cell–derived neural precursors[2]. In that study, neural differentiation was first observed in cultures grown for three weeks at a high density on a feeder layer by the appearance of areas containing cells with short processes that expressed PSA-NCAM. These cell clusters, identified by characteristic morphology within a mixture of differentiated ES cells, were then manually extracted with a micropipette and, upon replating in a serum-free medium, formed spherical structures. In contrast, our procedure permits efficient enzyme-based isolation of neuroepithelial cells generated in the presence of FGF-2. Whether the effect of FGF-2 observed in our system is primarily due to neural induction or stimulation of proliferation remains to be elucidated.

The chemically defined culture system described here provides an opportunity to explore the effects of single factors on human neuroepithelial proliferation and specification *in vitro*. Like precursors derived from the developing human brain, human ES cell–derived precursors show a strong proliferative response to FGF-2 (ref. 21). However, no additive or synergistic effects on proliferation can be elicited by EGF or LIF. This finding differs from data obtained with primary cells[14,18–20] and may suggest that proliferating ES cell–derived neural precursors represent a more immature stage than precursor cells derived from the fetal human brain. Studies on rodent cells indeed indicate that neural stem cells isolated during early neurogenesis depend on FGF-2 for proliferation and that the responsiveness to EGF is acquired only at later stages of neural precursor cell differentiation[25,26].

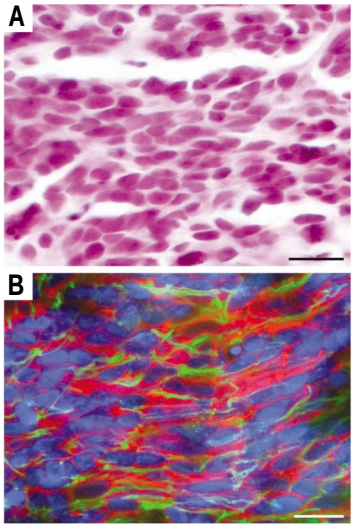

Figure 3. Clustered donor cells in the recipient ventricles. Upon transplantation into neonatal mice, the grafted cells form intraventricular clusters with primitive neuroepithelial morphology as shown by hematoxylin and eosin staining (A). (B) Clustered cells display immunoreactivity to nestin (green) and β_{III}-tubulin (red) antibodies. Nuclei are counterstained with Hoechst (blue). Bars = 20 μm.

Following transplantation into the neonatal mouse brain, the ES cell–derived neural precursors became incorporated into various brain regions, where they differentiated into neurons and glia. The failure to detect mature oligodendrocytes *in vivo* is probably due to the low oligodendroglial differentiation efficiency of human neural precursors compared with their rodent counterparts[22]. Remarkably, donor-derived neurons were not restricted to sites exhibiting postnatal neurogenesis but were also found in many other regions of the brain. Similar data were obtained in studies involving transplantation of human CNS-derived precursors into the adult rodent brain[23]. The engraftability of individual donor cells beyond the period of neurogenesis may point to a potential application of human ES cell–derived neural precursors in cell replacement in the adult CNS. More studies will be required to determine whether and to what extent the incorporated cells acquire region-specific properties and become functionally active.

With the exception of intraventricular clusters composed of mature and immature neuroepithelial cells, no space-occupying lesions were detected within the host brains. Most notably, no teratoma formation was observed during an eight-week postoperative period. While it is clear that more rigorous safety studies in nonhuman primates will be required before considering potential clinical applications, our data suggest that neural precursors isolated from differentiating human ES cell cultures represent a promising donor source for neural repair.

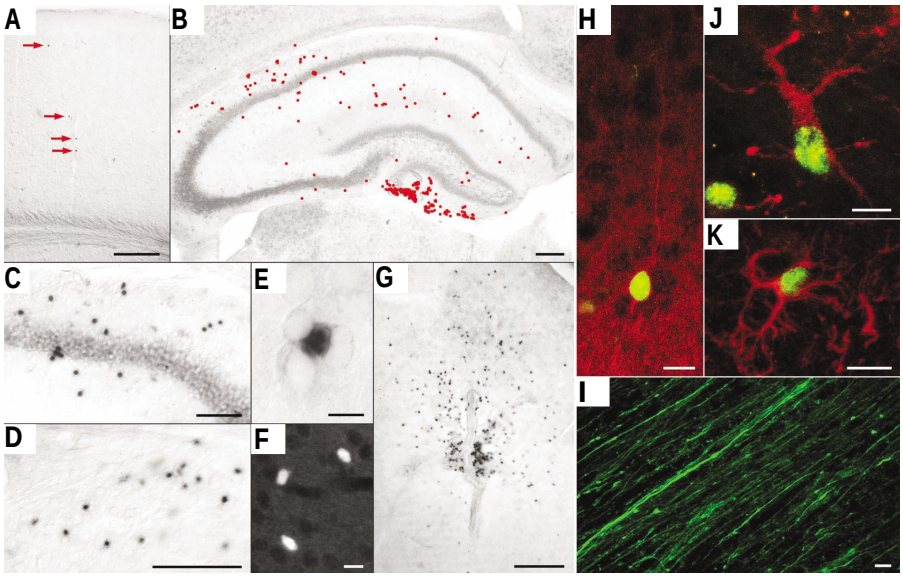

Figure 4. Incorporation and differentiation of ES cell–derived neural precursors *in vivo*. Grafted cells are detected by *in situ* hybridization with a probe to the human *alu* repeat element (A–E, G) or an antibody to a human-specific nuclear antigen (F). (A) Individual donor cells in the host cortex of an eight-week-old recipient (arrows). (B) Extensive incorporation of ES cell–derived neural precursors in the hippocampal formation. Cells hybridized with the human *alu* probe are color-coded with red dots. (C) Incorporated human cells in the vicinity of the hippocampal pyramidal layer at P14. (D) ES cell–derived cells in the septum of a four-week-old recipient mouse. (E) High-power view of an individual donor cell in the hypothalamus. Note the seamless integration between adjacent unlabeled host cells. (F) Donor cells in the striatum of a four-week-old host, detected with an antibody to a human-specific nuclear antigen. (G) Extensive migration of transplanted cells from the aqueduct into the dorsal midbrain. (H) Human ES cell–derived neuron in the cortex of a two-week-old host, exhibiting a polar morphology and long processes. The cell is double labeled with antibodies to a human-specific nuclear marker (green) and β_{III}-tubulin (red). (I) Network of donor-derived axons in the fimbria of the hippocampus, identified with an antibody to human neurofilament. (J) Donor-derived multipolar neuron, double labeled with antibodies recognizing the *a* and *b* isoforms of MAP2 (red) and human nuclei (green). (K) ES cell–derived astrocyte in the cortex of a four-week-old animal, double labeled with the human nuclear marker (green) and an antibody to GFAP (red). Note that all the double labelings are confocal images confirmed by single optical cuts. Bars: (A, B, G) 200 μm; (C, D) 100 μm; (E, F, H–K) 10 μm.

Experimental protocol

Culture of ES cells. ES cell lines, H1 (passages 16–33), H9 (passages 34–55), and a clonal line derived from H9, H9.2 (passages 34–46), were cultured on a feeder layer of irradiated mouse embryonic fibroblasts with a daily change of a medium that consisted of Dulbecco's modified Eagle's medium (DMEM)/F12, 20% serum replacement (Gibco, Rockville, MD), 0.1 mM β-mercaptoethanol, 2 μg/ml heparin, and 4 ng/ml FGF-2 (PeproTech Inc., Rocky Hill, NJ). The H9.2 clone was derived from H9 at passage 29 by plating individual cells under direct microscopic observation into single wells[4]. Its capacity for self-renewal and differentiation was similar to that of H9 after ~300 doubling times[4]. Karyotype analyses indicated that the lines at the given passages were diploid.

Differentiation cultures of ES cells. ES cell cultures were incubated with dispase (0.1–0.2 mg/ml; Gibco) at 37°C for 30 min, which removed ES cell colonies intact. The ES cell colonies were pelleted, resuspended in ES cell medium without FGF-2, and cultured for four days in a 25 cm^2 tissue culture flask (Nunclon, Roskilde, Denmark) with a daily medium change. ES cell colonies grew as floating EBs, while any remaining feeder cells adhered to the flask. The feeder cells were removed by transferring the EBs into a new flask. EBs (~50/flask) were then plated in a 25 cm^2 tissue culture flask (Nunclon) in DMEM/F12, supplemented with insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), sodium selenite (30 nM), and heparin (2 μg/ml) in the presence of FGF-2 (20 ng/ml)[14,15].

Isolation and culture of neural precursor cells: The differentiating EBs cultured for 8–10 days were incubated with 0.1 mg/ml dispase at 37°C for 15–20 min to separate the clusters of rosette cells from the surrounding flat cells. The rosette clumps retracted, whereas the surrounding flat cells

remained adherent. At this point, the rosette clumps were dislodged by swaying the flask, leaving the flat cells adherent. The clumps were pelleted, gently triturated with a 5 ml pipette, and plated into a culture flask for 30 min to allow the contaminating individual cells to adhere. The floating rosette clumps were then transferred to a new flask coated with poly-(2-hydroxyethyl-methacrylate) to prohibit attachment, and cultured in a medium used for human neural precursors[14] in the presence of FGF-2 (20 ng/ml). The cultures were split 1:2 or 1:4 every other week by triturating the neurospheres into smaller ones with a Pasteur pipette[14]. Freshly separated cell clusters and the flat cells left behind were dissociated with trypsin (0.025% in 0.1% EDTA) and counted to quantify the efficiency of neural differentiation and isolation. The percentage of putative neural precursors (rosette cells) among the total cells differentiated from ES cells was obtained based on three independent experiments on H9 and H9.2 lines. For analyses of the differentiation potential of the ES cell–derived neural precursors, cells were cultured on ornithine/laminin substrate in a medium consisting of DMEM/F12, N2 supplement (Gibco), cAMP (100 ng/ml), and brain-derived neurotrophic factor (BDNF, 10 ng/ml; PeproTech) in the absence of FGF-2. ES cell–derived neural precursors were cultured in DMEM supplemented with N1 (Gibco) and PDGF-A (2 ng/ml) as described[14] to promote oligodendrocyte differentiation. Morphological analyses and immunostaining with markers for progenitors and more mature neural cells were performed during the course of *in vitro* differentiation.

Histochemical and immunohistochemical staining. For morphological analysis of the rosette formations, cultures with rosettes were rinsed with PBS, fixed in 4% paraformaldehyde and 0.25% glutaraldehyde for 1 h, and embedded in plastic resin as described[15]. Sections of 1-μm thickness were stained with toluidine blue. Histochemical staining of alkaline phosphatase in differentiated EB cultures and ES cells (as a positive control) was performed using Vector Blue alkaline phosphatase staining kit (Vector Laboratories, Burlingame, CA). For immunostaining, coverslip cultures were incubated with anti-nestin (polyclonal, gift of R. McKay of NINDS, 1:1,000) (PSA-NCAM, mouse IgM, gift of G. Rougon of University of Marseilles, France, 1:200), anti-Musashi-1 (rat IgG, gift of H. Okano, University of Tokyo, Japan, 1:500), anti-GFAP (polyclonal, Dako, 1:1,000), anti-human GFAP (Sternberger Monoclonals, Baltimore, MD, 1:10,000), O4 (mouse IgM, hybridoma supernatant, 1:50), and anti-TH (Pel Freez, Rogers, AK; 1:500). Antibodies to β_{III}-tubulin (mouse IgG, 1:500), neurofilament (NF) 68 (mouse IgG, 1:1,000), NF 200 (polyclonal, 1:5,000), MAP2ab (mouse IgG, 1:250) (GABA, polyclonal, 1:10,000), and glutamate (mouse IgG, 1:10,000) were purchased from Sigma (St Louis, MO). Antigens were visualized using appropriate fluorescent secondary antibodies detailed elsewhere[14,15]. For analysis of BrdU incorporation, four coverslip cultures in each group were incubated with 2 μmol of BrdU for 16 h. The cultures were fixed in 4% paraformaldehyde, denatured with 1 N HCl, and processed for immunolabeling and cell counting[14,15]. Negative controls lacking the primary antibodies were included in each series.

Intracerebroventricular transplantation and *in vivo* analysis. Aggregates of ES cell–derived neural cells harvested either immediately after dispase-mediated isolation or within the first four passages of growth factor expansion were dissociated with trypsin (0.025% in 0.1% EDTA at 37°C for 5–10 min), passed through a 70 μm filter, and suspended in L15 medium (Gibco) at a concentration of 100,000 viable cells/μl. Using illumination from below the head, 2–3 μl of cell suspension was slowly injected into each of the lateral ventricles of cryoanesthetized newborn mice (C3HeB/FeJ). The grafted animals were immunosuppressed by daily injection of cyclosporin A (10 mg/kg, intraperitoneal). One, two, four, and eight weeks following transplantation, mice were perfused transcardially with Ringer's followed by 4% paraformaldehyde prepared in PBS. Brains were dissected and postfixed in the same fixative at 4°C until use. Donor cells were identified in 50 μm coronal vibratome sections by *in situ* hybridization using a digoxigenin-labeled probe to the human *alu* repeat element[24]. Alternatively, sections were subjected to microwave antigen retrieval (180 W in 0.01 M citrate buffer, pH 6.0, for 1 h) and incubated with an antibody to a human-specific nuclear antigen (MAB1281, Chemicon, Temecula, CA, 1:50) in the presence of 0.1% Triton X-100. Immunopositive cells were double labeled with antibodies to GFAP (1:100), nestin, β_{III}-tubulin (TUJ1, BabCo, Richmond, CA, 1:500), MAP2 (Sigma, clones AP-20 and HM-2, 1:300), and phosphorylated medium-molecular-weight human NF (clone HO-14, 1:50, a gift of J. Trojanowski). Antigens were detected by appropriate fluorophore-conjugated secondary antibodies[24]. Sections were analyzed on Zeiss Axioskop 2 and Leica TCS laser scan microscopes. Specificity of human cell markers was confirmed by the absence of signal in nontransplanted control animals. In addition, omission of the first antibody was used as a negative control.

Acknowledgments

We are grateful for the technical support provided by C. Daigh, A. Graf, J. Dean, and Z. Chang and for helpful comments from Clive Svendsen. This study was supported by the Myelin Project (Washington, DC) and the Consolidated Anti-Aging Foundation (Naples, FL).

Received 23 May 2001; accepted 4 October 2001

1. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
2. Reubinoff, B.E., Pera, M.F., Fong, C.-F., Trounson, A. & Bongso, A. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat. Biotechnol.* **18**, 399–404 (2000).
3. Thomson, J.A. & Odorico, J.S. Human embryonic stem cell and embryonic germ cell lines. *Trends Biotechnol.* **18**, 53–57 (2000).
4. Amit, M. *et al.* Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol*. **227**, 271–278 (2000).
5. Wiles, M.V. & Keller, G. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* **111**, 259–267 (1991).
6. Klug, M.G., Soonpaa, M.H., Koh, G.Y. & Field, L.J. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J. Clin. Invest.* **98**, 216–224 (1996).
7. Soria, B. *et al.* Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* **49**, 157–162 (2000).
8. Bain, G., Kitchens, D., Yao, M., Huettner, J.E. & Gottlieb, D.I. Embryonic stem cells express neuronal properties *in vitro*. *Dev. Biol.* **168**, 342–357 (1995).
9. Okabe, S., Forsberg-Nilsson, K., Spiro, A.C., Segal, M. & McKay, R.D.G. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells *in vitro*. *Mech. Dev.* **59**, 89–102 (1996).
10. Mujtaba, T. *et al*. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. *Dev. Biol.* **214**, 113–127 (1999).
11. Brustle, O. *et al*. Embryonic stem cell–derived glial precursors: a source of myelinating transplants. *Science* **285**, 754–756 (1999).
12. Brustle, O. *et al*. *In vitro*–generated neural precursors participate in mammalian brain development. *Proc. Natl. Acad. Sci. USA* **94**, 14809–14814 (1997).
13. McDonald, J.W. *et al*. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nat. Med.* **5**, 1410–1412 (1999).
14. Zhang, S.-C., Ge, B. & Duncan, I.D. Tracing human oligodendroglial development *in vitro*. *J. Neurosci. Res.* **59**, 421–429 (2000).
15. Zhang, S.-C., Ge, B. & Duncan, I.D. Adult brain retains the potential to generate oligodendroglial progenitors with extensive myelination capacity. *Proc. Natl. Acad. Sci. USA* **96**, 4089–4094 (1999).
16. Lendahl, U., Zimmerman, L.B. & McKay, R.D. CNS stem cells express a new class of intermediate filament protein. *Cell* **60**, 585–595 (1990).
17. Kaneko, Y. *et al*. Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev. Neurosci.* **22**, 139–153 (2000).
18. Svendsen, C.N., Clarke, D.J., Rosser, A.E. & Dunnett, S.B. Survival and differentiation of rat and human epidermal growth factor–responsive precursor cells following grafting into the lesioned adult central nervous system. *Exp. Neurol.* **137**, 376–388 (1996).
19. Carpenter, M.K. *et al*. *In vitro* expansion of a multipotent population of human neural progenitor cells. *Exp. Neurol.* **158**, 265–278 (1999).
20. Vescovi, A.L. *et al*. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp. Neurol.* **156**, 71–83 (1999).
21. Flax, J.D. *et al*. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat. Biotechnol.* **16**, 1033–1039 (1998).
22. Svendsen, C.N., Caldwell, M.A. & Ostenfeld, O. Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol.* **9**, 499–513 (1999).
23. Fricker, R.A. *et al*. Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J. Neurosci.* **19**, 5990–6005 (1999).
24. Brustle, O. *et al*. Chimeric brains generated by intraventricular transplantation with human brain cells into embryonic rats. *Nat. Biotechnol.* **16**, 1040–1044 (1998).
25. Kalyani, A.D., Hobson, K. & Rao, M.S. Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization and clonal analysis. *Dev. Biol.* **186**, 202–223 (1997).
26. Tropepe, V. *et al*. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol.* **208**, 166–188 (1999).