J Neurosurg 103:115–123, 2005

Embryonic stem cell–derived astrocytes expressing drug-inducible transgenes: differentiation and transplantion into the mouse brain

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Object. Embryonic stem cell (ESC)–derived astrocytes have many theoretical and practical advantages as vectors for delivery of gene therapy to the central nervous system (CNS). The aim of this study was to generate highly pure populations of ESC-derived astrocytes expressing drug-inducible transgenes, while minimizing contamination by undifferentiated ESCs

Methods. Embryonic stem cells carrying a doxycycline-inducible *green fluorescent protein (GFP)* transgene were induced to differentiate into astrocytes by using feeder cell–free conditions that are completely defined. More than 95% of these cells expressed the astrocyte markers glial fibrillary acidic protein and GLT-1 glutamate transporter, and the morphological characteristics of these cells were typical of astrocytes. The expression of additional astrocyte markers was detected using reverse transcription–polymerase chain reaction. Undifferentiated ESCs comprised fewer than 0.1% of the cells after 10 days in this culture. Positive and negative selection techniques based on fluorescence-activated cell sorting were successfully used to decrease further the numbers of undifferentiated ESCs. Fully differentiated astrocytes expressed a *GFP* transgene under the tight control of a doxycycline-responsive promoter, and maintained their astrocytic phenotype 24 hours after transplantation into the mouse brain.

Conclusions. This study shows that transgenic ESCs can be induced to differentiate into highly pure populations of astrocytes. The astrocytes continue to express the transgene under the tight control of a drug-inducible promoter and are suitable for transplantation into the mouse brain. The number of potentially hazardous ESCs can be minimized using cell-sorting techniques. This strategy may be used to generate cellular vectors for delivering gene therapy to the CNS.

KEY WORDS • embryonic stem cell • astrocyte • gene therapy • transplantation • teratoma

GENE therapy strategies currently studied in clinical trials involve the use of viral vectors to deliver therapeutic transgenes directly to normal and tumor cells within the CNS.[4,11,18] Viral vectors have a number of theoretical and practical limitations, including limited diffusion into the brain parenchyma, poor transfection of some cell types, antigenicity, and, for retroviral and adeno-associated viral vectors that incorporate into the host chromosome, insertional mutagenesis with the potential to cause tumors.[21,28,32] The use of genetically modified cells to deliver gene therapy to the CNS may avoid some of these limitations. Astrocytes may be ideally suited for this purpose.[36] Astrocytes are native to the CNS, which should maximize their survival and function after transplantation. They normally provide trophic and tropic support to neurons and play important roles in protecting neurons from toxic levels of glutamate and potassium;[9] therefore, transplanted astrocytes should be innocuous or protective within the host brain. Astrocytes are highly secretory types of cells and are able to generate large amounts of transgenic protein. Finally, normal astrocytes have the ability to migrate along white matter tracts after transplantation into the brain;[16,39] this migratory capacity may be useful for delivery of gene therapy to infiltrative tumors such as malignant gliomas.

Transgenic astrocytes suitable for use as gene therapy vectors could be derived from a number of sources. Autologous astrocytes or pluripotent neural precursor cells from the subventricular zone may be obtained by performing a biopsy, and then expanded in vitro and transfected with viral vectors to express therapeutic genes. Fetal tissues could provide a third source of astrocytes for transplantation. These approaches are limited by the use of potentially hazardous viral vectors, the need for multiple surgical procedures or large amounts of fetal tissue, and the restricted proliferative

Abbreviations used in this paper: CNS = central nervous system; DAPI = 4′,6-diamidino-2-phenylindole-2HCl; EB = embryoid body; EFC = embryonic feeder cell; ESC = embryonic stem cell; FACS = fluorescence-activated cell sorting; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; IMDM = Iscove modified Dulbecco medium; MTG = methyl β-D-thiogalactoside; NCAM = neural cell adhesion molecule; NPC = neural progenitor cell; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PDGF = platelet-derived growth factor; RT = reverse transcription.

capacity of differentiated neural cell types. Embryonic stem cells could provide an alternative source of transgenic astrocytes that circumvents these difficulties. These cells are totipotent cells obtained from the inner cell mass of the embryo while in the blastocyst stage.[20] They have unlimited proliferative capacity and, unlike other cell types, can be genetically modified using homologous recombination, eliminating the need for viral vectors and permitting multiple, precisely determined genetic modifications. Wild-type and genetically modified ESCs have been tested in several animal models of CNS disease, including demyelinating disease,[7,27] trauma,[29] and Parkinson disease.[2,5,25] The use of ESCs for transplantation purposes has disadvantages, including regulatory constraints[40] and the possibility of teratoma formation by undifferentiated ESCs.[1,5,15,22,42] We describe a method of generating highly pure cultures of astrocytes from mouse ESCs that has advantages over previously described protocols including a very high yield of astrocytes, completely defined culture media, and differentation in the absence of EFCs. We demonstrate highly regulated, robust drug-inducible expression of a transgene following insertion via homologous recombination.

We describe methods for minimizing the contamination of ESC-derived astrocytes by undifferentiated ESCs. Finally, we show that transgenic ESC-derived astrocytes are suitable for transplantation into the mouse brain.

Materials and Methods

Mouse ESC Culture

The mouse cells used in this study, Ainv-GFP ESC, a gift from M. Kyba,[26] contain two transgenes inserted by site-specific homologous recombination: a constitutively expressed gene encoding a tetracycline/doxycycline–binding, transcriptional activator fusion protein, and a *GFP* reporter gene downstream from a tetracycline-response element. The cells were maintained at all times in an incubator containing a humidified atmosphere of 95% air/5% CO_2 at 37°C. Undifferentiated Ainv-GFP cells were propagated on irradiated mouse feeder cells in a manner that has previously been described.[1] For astrocyte differentiation, the Ainv-GFP cells were passaged twice on gelatin-coated plates to remove the feeder cells, after which they were trypsinized, washed with IMDM containing 15% FCS (Gemini Bio-Products, Woodland, CA), and seeded at a quantity ranging from 10^4 to 3×10^4 cells/ml in 60-mm cell-culture plates in StemPro-34 medium (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine (Gibco), penicillin–streptomycin (Gibco), 4.5×10^4 M MTG (Sigma Chemical Co., St. Louis, MO), 0.5 mM ascorbic acid (Sigma Chemical Co.), and 2% Kit ligand.[17] After 2 days, small EBs had formed, and these were transferred to IMDM that was supplemented with 15% serum replacement (Gibco), 2 mM L-glutamine, penicillin–streptomycin, 4.5×10^4 M MTG, and 0.5 mM ascorbic acid. After 4 more days, the larger EBs were allowed to settle out of suspension and were transferred to gelatin-coated tissue culture dishes in IMDM that was supplemented with 15% serum replacement, 20 ng/ml epidermal growth factor (R&D Biosystems, Minneapolis, MN), 10 ng/ml human fibroblast growth factor–2 (R&D Biosystems), and 1 μg/ml laminin (Sigma Chemical Co.).[34] The medium in each dish was changed every other day. After 5 days, the medium was changed to IMDM that was supplemented with 15% serum replacement, 10 ng/ml human fibroblast growth factor–2, and 10 ng/ml PDGF-AA or PDGF-BB (R&D Biosystems).[34] When appropriate, 1 μg/ml doxycycline (Sigma Chemical Co.) was added to the medium at least 24 hours before an analysis of *GFP* expression was undertaken.

The RT-PCR Study

Total cellular RNA from cultured cells or freshly dissected mouse brain was purified using the RNeasy kit (Qiagen, Inc., Valencia, CA), with an on-column DNAse treatment. All RNA preparations were spectrophotometrically quantified and examined for degradation by using gel electrophoresis before RT. Reverse transcription was performed on 1 μg of total RNA by using the OmniScript reverse transcriptase kit (Qiagen, Inc., Valencia, CA) and PDN6 random hexamer primers (Pharmacia, Inc., Piscataway, NJ) in a total volume of 20 μl. Each 12.5-μl PCR mixture contained a 0.5-μl complementary DNA template. All PCR amplifications were performed for 35 cycles (the primer sequences and the annealing temperatures have previously been published[12,19,41,44]). The PCR products were examined on 1.8% agarose gels and photographed using an Eagle Eye II imager (Stratagene, La Jolla, CA).

Immunofluorescence Labeling

The differentiated cells were grown on glass coverslips (Fisher Scientific, Hampton, NH) coated with poly-D-lysine (Sigma Chemical Co.), whereas ESCs were grown on glass coverslips coated with gelatin (Sigma Chemical Co.). For immunofluorescence labeling, the cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature, washed again, and preincubated at room temperature in 10% normal goat serum (Sigma Chemical Co.) for 30 minutes and then in primary antibody for 1 hour. The primary antibodies that we used and their respective dilutions are as follows: rabbit anti-GFAP, 1:100 (Sigma Chemical Co.); monoclonal anti-GFP, 1:200 (Molecular Probes, Eugene, OR); monoclonal anti–maltose binding protein, antinestin, and anti–β_{III}-tubulin, 1:100 each (Chemicon, Temecula, CA); monoclonal anti–E-cadherin, 1:300; monoclonal anti–Oct-3/4, 1:100; and FITC-conjugated rat anti–CD31, 1:100 (Pharmingen, San Diego, CA). The cells were then washed and incubated in appopriate secondary antibodies for 1 hour at room temperature. The secondary antibodies that we used and their respective dilutions are as follows: Cy3-conjugated goat anti–rabbit, 1:800; anti–mouse and anti–rat, 1:500 each; and FITC-conjugated goat anti–rat, 1:200 (all from Pharmingen). The cells were then washed and mounted in Vectashield with added DAPI (Vector Laboratories, Burlingame, CA), and examined by performing fluorescence microscopy with the Openlab imaging system (Improvision, Lexington, MA).

Fluorescence Activated Cell Sorting

Cells growing in a monolayer or in suspension as EBs were trypsinized and triturated through a 19-gauge needle. Trypsinization was stopped with an equal volume of FCS. The cells were then centrifuged, resuspended in washing buffer (PBS supplemented with 10% FCS and 0.01% NaN_3), and counted. To perform FACS by using antibodies to intracellular proteins, the cells were permeabilized and treated according to previously published protocols.[43] To perform FACS by using antibodies to cell surface antigens, the cells were distributed into wells of a 96-well plate, sequentially labeled with primary antibodies and secondary antibodies or detection reagents as listed later, and counted with the aid of a fluorescence-activated cell sorter (FACScalibur; Becton Dickinson, San Jose, CA) by using the Cellquest software package (BD Biosciences, San Jose, CA). Gating with an appropriate measurement of forward and side scatter was used to exclude dead cells and cell aggregates from the sorting, and 10,000 cells were counted per analysis. Cell sorting and recovery was performed with the aid of a MoFlo cell sorter (DakoCytomation, Fort Collins, CO). Reagents used for cell labeling and sorting, and their respective dilutions, included the following: monoclonal rat anti–E-cadherin, 1:300; biotinylated monoclonal rat anti–CD31, 1:100; phycoerythrin-conjugated anti–rat, 1:300; and Cy5-phycoerythrin–conjugated streptavidin, 1:200 (all from Pharmingen).

Allotransplantation of Astrocytes Into the Mouse Brain

All protocols in which live animals were used were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. The ESC-derived astrocyte cultures were trypsinized, washed, counted, and resuspended at 3×10^4 cells/μl in IMDM. The cells were loaded into a Hamilton syringe, which was mounted on a stereotactic animal frame (Stoelting, Wood Dale, IL).

After anesthesia had been induced the syngeneic 129/Ola mice (Harlan, Indianapolis, IN), each between 3 and 6 weeks of age, scalp incisions were made and burr holes were drilled anterolaterally to the bregma. The mice were then placed in the stereotactic frame, and 1 μl of cell suspension was slowly injected into either the right corpus callosum (four animals) or the right striatum (four animals) by following these coordinates: 0.62 mm anterior to the bregma, 2 mm lateral, and 3.5 mm deep to the cortical surface (striatum), and 2.5 mm deep to the cortical surface (corpus callosum).[31] Sham injections of IMDM were made into the corresponding left corpus callosum or striatum in the same animals. The injection sites were marked on the cortical surface by using Pelikan black ink, and the incisions were closed. The viability of the transplanted cells was checked by recovering any unused cells from the Hamilton syringe, staining them with vital dye, and allowing them passage in culture.

Immunohistochemical Analyses of Mouse Brains

At various time points, the animals were anesthetized and intracardially perfused with cold 4% paraformaldehyde in PBS. The animals' brains were removed whole and postfixed in 4% paraformaldehyde in PBS. The injection sites were identified, and coronal slices centered on the injection sites were selected for sectioning. Coronal 2-mm-thick slices were paraffin embedded and sliced into 5-μm serial sections by using a microtome. Slices were stained with H & E and selected slices were deparaffinized in xylene and ethanol, treated with hydrogen peroxide to eliminate endogenous peroxidases, and labeled with polyclonal anti-GFAP (dilution 1:200; Dako Corp., Carpinteria, CA) and the ABC detection kit with diamino benzidine substrate (Vector Laboratories). Negative control sections were incubated with secondary antibody alone. Stained sections were mounted in Permount and examined with the aid of a microscope.

Results

Generation of Astrocytes From Mouse ESCs

After 6 days of differentiation in serum-free suspension cultures, the EBs were plated either intact, as partially dissociated aggregates, or as a single-cell suspension onto gelatin, fibronectin, or poly-D-lysine–coated tissue-culture dishes in medium either containing or not containing MTG and ascorbic acid. The cultures were monitored for cell viability and growth and analyzed for expression of neural cell type–specific markers between 7 and 10 days following plating. After plating, the cells formed clusters of small, round cells, and flat or spindle-shaped cells began to migrate away from these clusters and form confluent sheets. Confluence was usually achieved by Day 7 following plating; approximately 3×10^6 cells were generated from a starting ESC population of between 10^4 and 3×10^4 cells. Similar rates of cell growth and yields of cells with astrocyte characteristics were observed regardless of whether the EBs were plated whole, partially dissociated, or completely dissociated. The use of gelatin or fibronectin substrate did not affect cell growth and differentiation, although cell growth was markedly slower when the EBs were plated on poly-D-lysine–coated dishes. The proportion of astrocytes remained the same when either PDGF-AA or PDGF-BB was included in the culture medium, but was somewhat higher when MTG and ascorbic acid were omitted. Similar results were obtained using two other ESC lines (data not shown).

Nine days after plating, up to 98% of the cells were immunoreactive with antibodies to GFAP, which was demonstrated by microscopic analysis (Fig. 1 *upper*) and FACS (Fig. 1 *lower*). The exact proportion of GFAP-positive cells depended on whether polyclonal or monoclonal antibodies had been used, which is typical of astrocytes in vivo and in culture. Cells growing at a low density demonstrated either stellate or compact shapes that were suggestive of type II and type I astrocytes, respectively. Some nonastrocytic cell types, including ependymal cells, also express GFAP. We evaluated expression of other astrocyte-selective markers, including the GLT-1 glutamate transporter and S100β protein.[33] The RT-PCR analysis showed that GFAP, GLT-1, and S100β were expressed in our astrocyte cultures but not in undifferentiated Ainv-GFP ESCs (Fig. 2 *left*). Immunofluorescence microscopy revealed that nearly all of the cultured astrocytes expressed GLT-1 (Fig. 2 *right*). Both RT-PCR and immunofluorescence staining confirmed that a small percentage of cells represented other neural cell types, including oligodendrocytes, neurons, and nestin-positive neural precursor cells (data not shown).

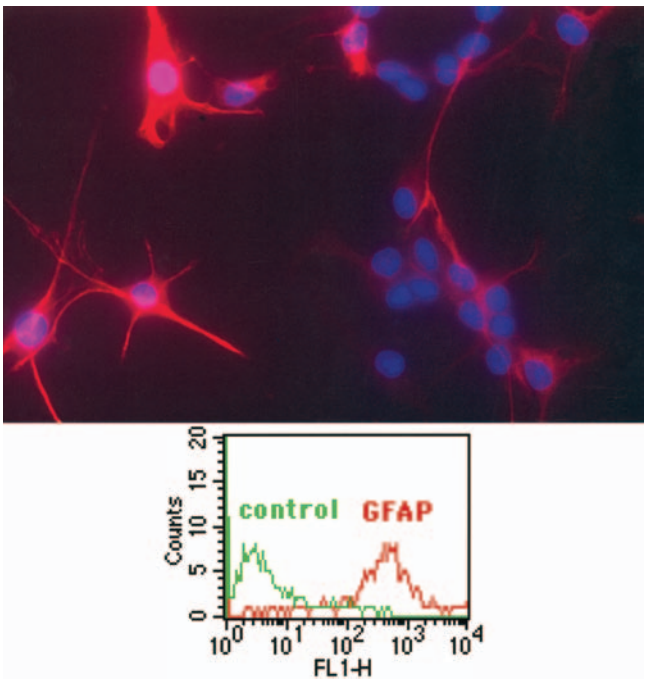

FIG. 1. Morphological characteristics and GFAP expression of ESC-derived astrocytes. *Upper:* Photomicrograph of ESC-derived astrocytes demonstrating antibodies to GFAP (red) and DAPI nuclear counterstaining (blue). Labeling with polyclonal anti-GFAP reveals the typical shapes of stellate and compact astrocytes in differentiated cultures. Original magnification × 150. *Lower:* Graph showing the results of an FACS analysis of cultured astrocytes labeled with polyclonal anti-GFAP demonstrating that 98% of the cells stained positively for GFAP. Controls were labeled with secondary antibody only.

Selective Strategies Minimize Contamination by Undifferentiated ESCs

Several strategies for minimizing contamination of our differentiated cells by residual ESCs were tested. First, we established efficient protocols for generating highly differentiated neural cells. The FACS analysis (Fig. 3) showed that approximately 1% of cells expressed murine ESC markers (CD31 and E-cadherin) after 7 days of differentia-

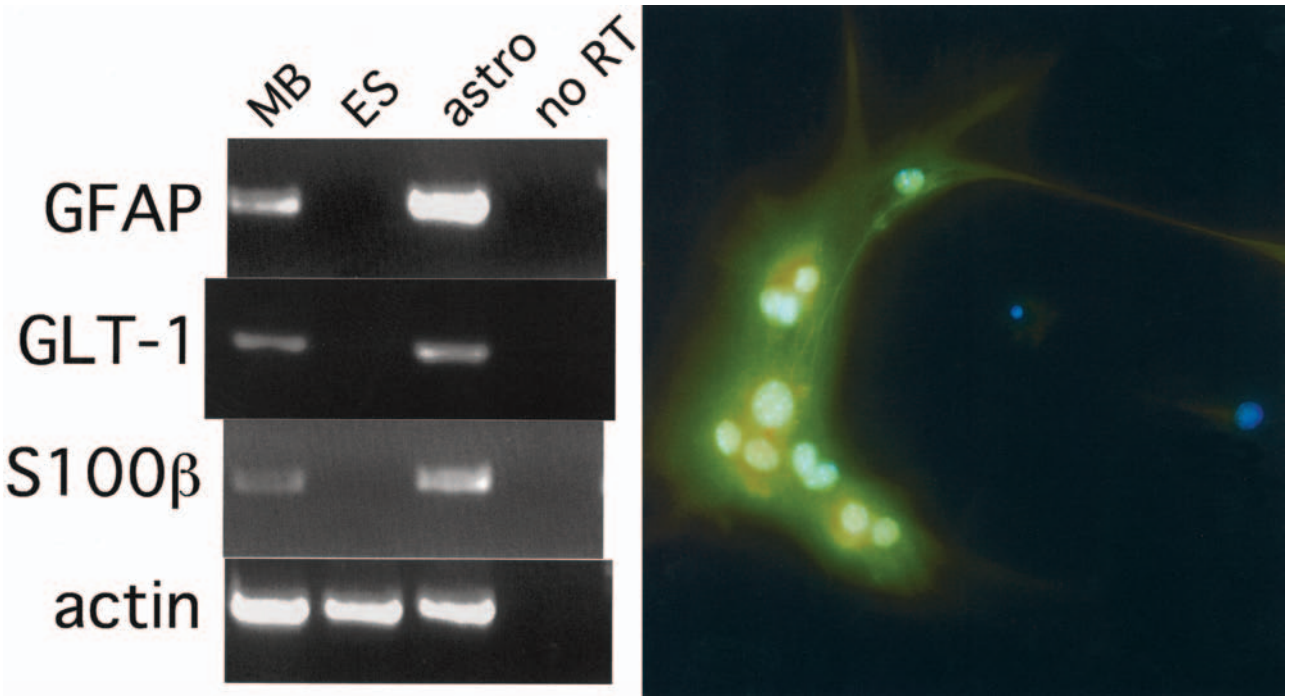

FIG. 2. Expression of other cell type-specific markers by ESC-derived astrocytes. *Left:* Results of an RT-PCR analysis of expression of the astrocyte-specific markers GFAP, GLT-1, and S100β protein showing gene expression in the whole mouse brain (MB) and in ESC-derived astrocytes (astro), but not in undifferentiated cultures of ESCs (ES) or astrocyte extracts not treated with reverse transcriptase (no RT). *Right:* Photomicrograph obtained by performing immunofluorescence microscopy with an antibody to GLT-1 (green) demonstrating that nearly all cells express GLT-1. The nuclei have been counterstained with DAPI (blue). Original magnification × 1000.

tion. After 10 days, fewer than 0.15% of cells expressed these ESC markers. Both RT-PCR and the immunofluorescence analysis of cultures at Day 15 confirmed the presence of small numbers of cells that expressed ESC markers including the transcription factors Oct-4, Rex-1, and nanog, and the cell surface marker E-cadherin. Following 2 or 3 weeks of culture, as proliferation of mature astrocytes slowed, the small number of residual ESCs in our cultures proliferated rapidly, resulting in large ESC colonies that overgrew the cultures.

Second, two FACS-based strategies for negative depletion of ESCs or positive selection of neural cells were used. For negative depletion, cells expressing either E-cadherin or CD31 were removed from cultures between Day 10 and Day 15 by using FACS. The remaining "double-negative" cells were subcultured for an additional 1 to 2 weeks and were monitored for ESC growth. For positive selection, we attempted to select neural cells by trypsinizing our cultures at Day 10, labeling with antibody to NCAM, and selecting only NCAM-positive cells for subculture. A few ESC colonies appeared early in both cultures and were removed manually by using a pipette. The initial RT-PCR analysis of sorted and unsorted cultures showed that low expression levels of the ESC marker genes *oct-4* and *nanog* persisted, indicating the presence of contaminating ESCs (Fig. 4). The ESC colonies appeared in both sorted and unsorted cell cultures after 16 days in culture; however, cultures of sorted cells had markedly fewer and smaller ESC colonies compared with those of unsorted cells after 3 weeks.

Astrocytes Derived From Ainv-GFP ESCs Continue to Express Transgene After Differentiation

The Ainv-GFP ESC line used in our study includes a constitutively active doxycycline-binding protein–transactivator fusion gene and the *GFP* reporter gene under the control of a doxycycline-responsive element. Adoption of fully differentiated phenotypes by cells expressing transgenes is sometimes associated with transcriptional silencing and a loss of transgene expression. To test whether mature Ainv-GFP–derived astrocytes are still able to express the transgene, we allowed our astrocytes to differentiate for 17 days in culture. Control cells not treated with doxycycline displayed minimal expression of *GFP* microscopically (Fig. 5 *upper*) and by FACS analysis (Fig. 5 *lower left*); in contrast, robust expression of *GFP* was seen in approximately 60% of mature cells after 24 hours of doxycycline treatment (Fig. 5 *center* and *lower right*). After exposure to doxycycline ESC–derived astrocytes expressed GFP and GFAP (Fig. 6).

Embryonic Stem Cell–Derived Astrocytes Maintain Their Phenotype 24 Hours After Transplantation Into the Mouse Brain

We performed experiments to test the suitability of ESC-derived astrocytes for transplantation into the mouse brain. Twenty-four hours after transplantation of 3×10^4 ESC-derived astrocytes into the striatum or corpus callosum of syngeneic mice, we observed maintenance of GFAP expression in the transplanted cell population (Fig. 7).

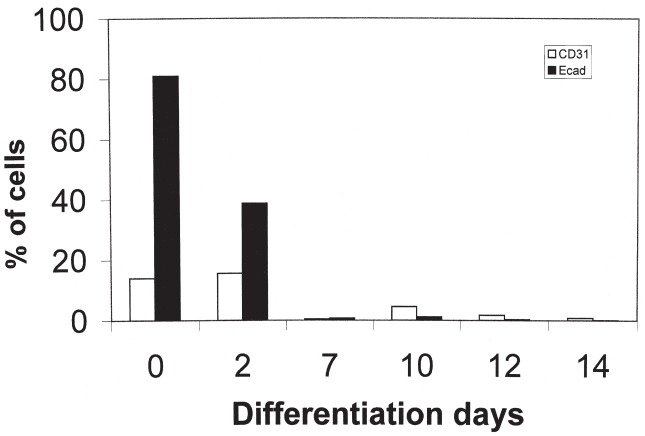

FIG. 3. Bar graph demonstrating expression of the ESC surface markers, E-cadherin (Ecad) and CD31, in differentiating astrocyte cultures during directed differentiation of ESCs into astrocytes. By 7 days in culture, only 1% of the cells are ESCs (double-positive staining). By Day 14, fewer than 0.1% are ESCs.

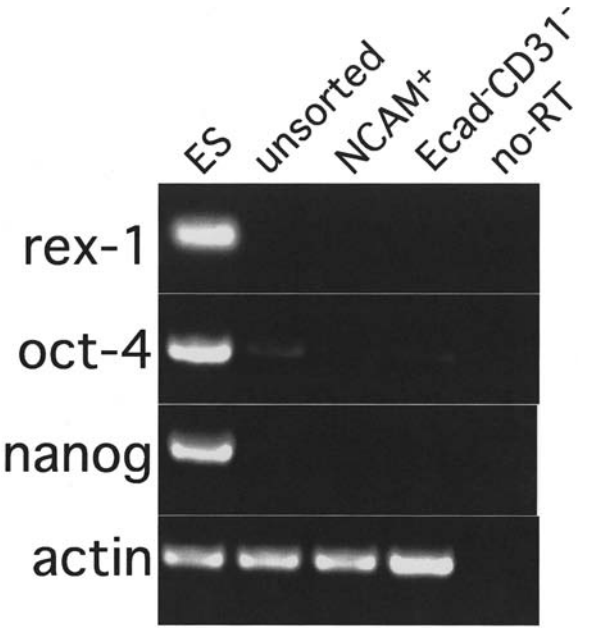

FIG. 4. Gel electrophoresis of RT-PCR products showing robust expression of the ESC markers rex-1, oct-4, and nanog in undifferentiated ESCs (ES). Expression is greatly diminished after differentiation, even without positive or negative (unsorted) selection. Low-level expressions of the *oct-4* and *nanog* genes persist in cultures generated from NCAM-positive cells ($NCAM^+$) or E-cadherin–CD31 double-negative cells ($Ecad^-CD31^-$). Cells not treated with reverse transcriptase (no-RT) provide a negative control.

Discussion

Unique Advantages of ESC-Derived Astrocytes as CNS Gene Therapy Vectors

Transplantation of genetically engineered neural cells to deliver gene therapy to the CNS shows promise in treating diseases such as brain tumor[3,13] and Parkinson disease.[30] Embryonic stem cell–derived astrocytes may be ideal gene therapy vectors for several types of CNS diseases. Astrocytes have potential advantages over other types of genetically engineered neural cells, such as NPCs. First, astrocytes are fully differentiated, whereas pluripotent NPCs theoretically can differentiate into functional neurons, which could interfere with existing neural circuits. Second, certain diseases, including Alexander disease[6] and mesial temporal lobe epilepsy,[14] may result from defects in astrocyte function or gene expression. Transplantation of genetically modified astrocytes may provide specific treatment for these disorders. Derivation of astrocytes from ESCs provides at least two additional advantages. First, unlike NPCs, ESCs can be permanently genetically modified using homologous recombination rather than potentially hazardous or transiently expressed viral vectors. Second, ESCs have an unlimited proliferative capacity; if stocks of undifferentiated human ESCs of various types of human leukocyte antigen are established in the future, an unlimited supply of immunologically matched neural cells for transplantation would be available.

Generation of Highly Pure Astrocyte Cultures Under Defined Feeder Cell–Free Conditions

Several groups have previously described the directed differentiation of ESCs into astrocytes.[2,34,38] In this report, we describe a protocol for generation of astrocytes from mouse ESCs that has significant advantages over earlier protocols. First, all the media used in cell differentiation are defined, eliminating the variables introduced with the use of cell line–conditioned media[34] or coculture with EFCs.[2] Second, we report a high yield of astrocytes and provide a detailed description of astrocyte-specific gene expression. Authors of earlier reports have accepted GFAP expression alone as presumptive evidence of astrocytic differentiation.[2,34] Whereas GFAP is the best single marker for astrocytes, other neural and nonneural cell types may also produce GFAP. Scheffler, et al.,[38] performed comprehensive and elegant histological and physiological studies that demonstrated astrocyte differentiation from ESCs; however, only moderate yields of astrocytes were obtained using their differentiation protocol. We obtained a greater than 95% yield of astrocytes, with coexpression of several astrocyte markers and typical morphological characteristics. Third, mouse EFCs are not used in our differentiation protocol. Embryonic feeder cells are mesenchymal cells obtained from mouse embryos; maintenance of mouse or human ESCs in the totipotent undifferentiated state is facilitated by a coculture with EFCs, which secrete undefined substances that help maintain the undifferentiated state. One recently described protocol for directed differentiation into neural cell types requires further coculture with EFCs.[2] There is widespread consensus among transplantation biologists that human ESCs cocultured with mouse EFCs cannot be used in future clinical trials, given the risk of transmission of mouse retroviruses. Although the development of human feeder cell lines could represent one solution to this problem,[23,35] finding ways to eliminate the need for feeder cells would be preferable. Although we maintained undifferentiated ESCs with EFCs for convenience, many laboratories routinely maintain undifferentiated mouse and human ESC lines without relying on a coculture with EFCs.[8,37]

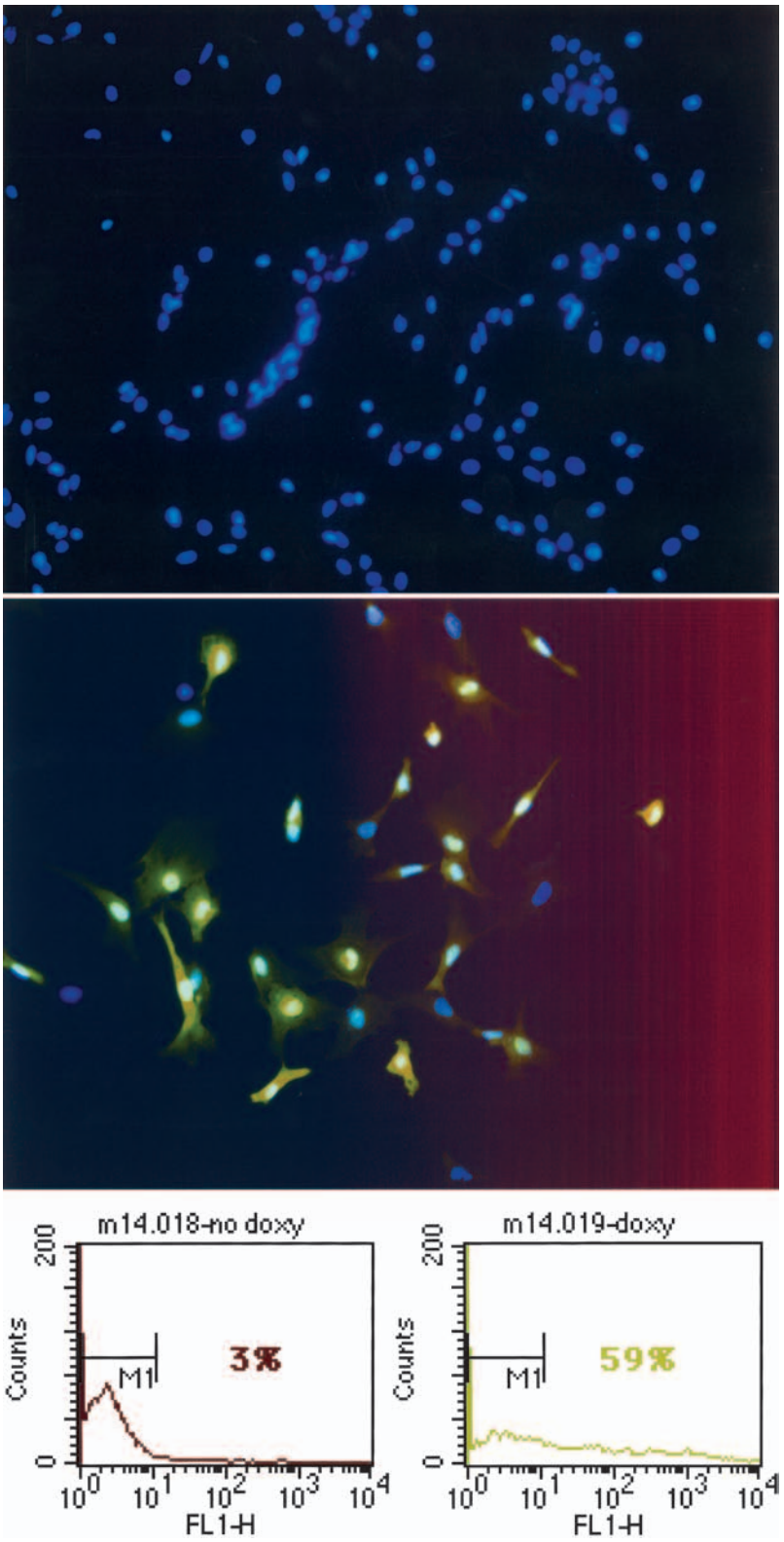

FIG. 5. Doxycycline-regulated expression of *GFP* transgene expression in ESC-derived astrocytes. *Upper* and *Center*: Photomicrographs obtained using fluorescence microscopy, demonstrating minimal *GFP* expression in astrocytes not treated with doxycycline *(upper)* and robust expression of *GFP* in astrocytes treated for 24 hours with doxycycline *(center)*. Original magnification × 200. *Lower Left* and *Right*: Results of FACS analyses showing minimal *GFP* expression in astrocytes not treated with doxycycline *(lower left)* and GFP expression in nearly 60% of cells treated with the tetracycline derivative *(lower right)*.

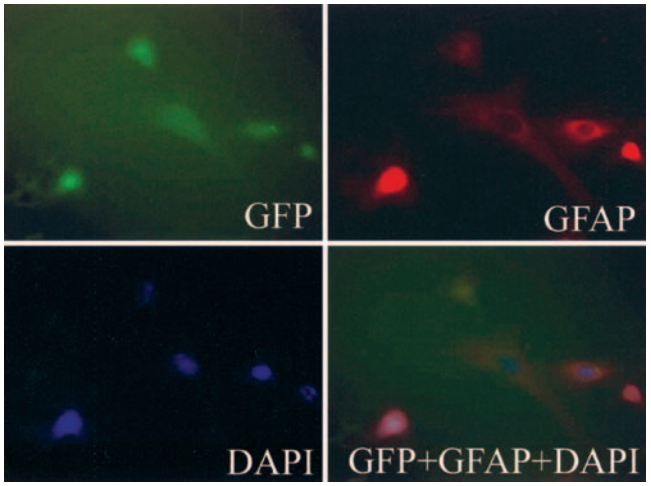

FIG. 6. Expression of GFP and GFAP in the same ES-derived astrocytes. Photomicrographs showing immunofluorescent stains in ESC-derived astrocytes: GFP alone with green stain *(upper left)*, GFAP alone with red stain *(upper right)*, DAPI counterstain without primary antibody *(lower left)*, and addition of GFP and GFAP stains in the same cells (double labeling).

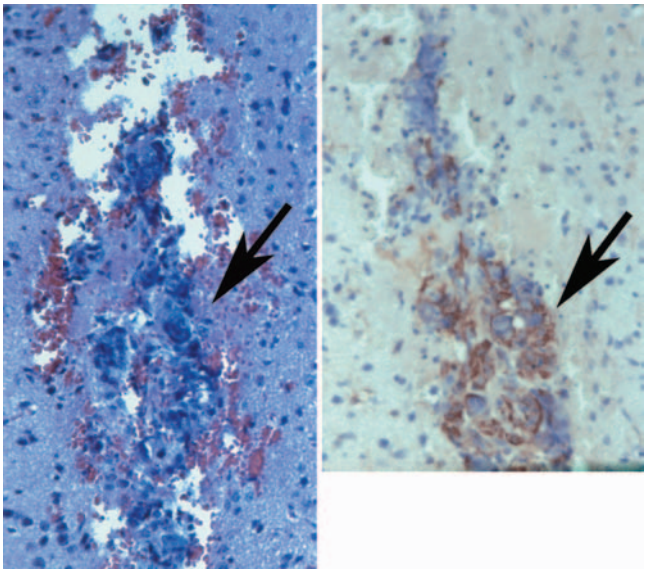

FIG. 7. Maintenance of the phenotype after transplantation of ESC-derived astrocytes into the mouse brain. Photomicrographs of 5-μm-thick coronal mouse brain sections obtained 24 hours after allotransplantation of ESC-derived astrocytes showing surviving ESC-derived astrocytes *(arrows)* continuing to express GFAP. H & E *(left)* and anti-GFAP *(right)*, original magnification × 200.

Significance of Residual ESCs in Neural Cultures for Transplantation

Experimental allotransplantation of undifferentiated ESCs into the brain or other tissues results in teratoma formation in recipient animals.[1,5,15,22,42] Xenotransplantation of ESCs is not associated with teratoma formation. The reasons for this lack of teratoma formation following xenotransplantation are unclear but are not due to immune factors, because immunosuppression of hosts does not permit teratoma formation when ESCs are transplanted from other species.[15] Predifferentiation of ESCs into neural or other cell types prevents teratoma formation in some experimental models of allotransplantation[2,10] but not in others.[15,22] As few as 50 undifferentiated ESCs remaining in a culture of ESC-derived neural cells may be sufficient to induce teratoma formation in recipient animals.[15]

We show that, despite a highly efficient protocol for generating mature astrocytes from ESCs, a small population of undifferentiated ESCs remains in the culture. The presence of undifferentiated ESCs is occult at first, but as cell division of mature astrocytes slows, the few remaining ESCs divide rapidly and overgrow the culture. Immunoisolation using panning has been used to generate neuron-enriched cultures from ESC-derived cells.[24] We describe FACS-based strategies for both negative and positive selection of mixed cultures of neural cells and ESCs, with significant decreases in ESC contamination following selection. Nevertheless, our experiments show that despite these strategies, contamination by residual ESCs persists and may not become apparent in culture for several weeks. For this reason, we conclude that in future experiments investigators must assess for ESC contamination not only in short-term cultures of purified cells, but also in long-term cultures. Ongoing experiments in our laboratory include more sophisticated sorting strategies and modification of culture conditions to eliminate residual ESCs.

Transgene Expression Persists in Differentiated Astrocytes and is Tightly Regulated

Transgene expression by astrocytes treated with viral vectors, including retroviral vectors, can be transient after transplantation into the CNS. We previously observed that transgene expression from ESCs containing the doxycycline-inducible transgene system was robust during early differentiation, but sometimes diminished after further differentiation into mature cell types in culture (V. Chen, unpublished observations). We found that fully differentiated astrocytes continue to express a *GFP* transgene under the control of the doxycycline-responsive promoter, but the percentage of the mature cells that express GFP is lower than the percentage of undifferentiated ESCs that express GFP. This phenomenon may be due to transcriptional silencing of genes in well-differentiated cell types. In the absence of doxycycline, few cells express *GFP*. This absence of "leaky" transgene expression is advantageous for therapeutic transgenes that could be hazardous if expressed in an uncontrolled fashion.

Transplantation of ESC-Derived Astrocytes Into the Mouse Brain

We studied the behavior of our ESC-derived astrocytes following allotransplantation into the mouse brain. We intended to assess survival, astrocyte-specific gene expression, migratory behavior, transgene expression, and the safety of our cells in vivo. We have shown that at 24 hours, transplanted ESC-derived astrocytes survive, remain in clusters at the implantation site, and continue to express the astrocyte marker GFAP. Potential benefits of ESC-derived astrocytes include the migratory characteristics of these cells. This makes these astrocytes highly desirable

to target diseases outside the implant site. Limitations include the possibility of inducing "astrocytic scars," producing molecules that could impede neuronal recovery and regeneration. We hope that our ongoing studies on the long-term behavior of transplanted ESC-derived astrocytes after allotransplantation into the mouse brain will bring insights into these issues in the near future.

Conclusions

Genetically modified mouse ESCs can be differentiated into highly pure cultures of astrocytes by using defined culture conditions and without the use of feeder cells. These astrocytes have the normal morphological characteristics of astrocytes and display the appropriate marker expression, and they continue to express transgenes in a robust but controlled manner. Selection techniques can minimize the number of undifferentiated ESCs that accompany ESC-derived astrocytes after differentiation in culture. These cells can be transplanted into the brain and may have unique advantages as vectors for delivery of gene therapy to treat CNS disease.

References

1. Asano T, Ageyama N, Takeuchi K, Momoeda M, Kitano Y, Sasaki K, et al: Engraftment and tumor formation after allogeneic in utero transplantation of primate embryonic stem cells. **Transplantation 76:**1061–1067, 2003
2. Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, et al: Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. **Nat Biotechnol 21:**1200–1207, 2003
3. Benedetti S, Pirola B, Pollo B, Magrassi L, Bruzzone MG, Rigamonti D, et al: Gene therapy of experimental brain tumors using neural progenitor cells. **Nat Med 6:**447–450, 2000
4. Betchen SA, Kaplitt M: Future and current surgical therapies in Parkinson's disease. **Curr Opin Neurol 16:**487–493, 2003
5. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, et al: Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. **Proc Natl Acad Sci USA 99:**2344–2349, 2002
6. Brenner M, Johnson AB, Boespflug-Tanguy O, Rodriguez D, Goldman JE, Messing A: Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. **Nat Genet 27:**117–120, 2001
7. Brustle O, Jones KN, Learish RD, Karram K, Coudhary K, Wiestler OD, et al: Embryonic stem cell-derived glial precursors: a source of myelinating transplants. **Science 285:**754–756, 1999
8. Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, et al: Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. **Dev Dyn 229:** 243–258, 2004
9. Chen Y, Swanson RA: Astrocytes and brain injury. **J Cereb Blood Flow Metab 23:**137–149, 2003
10. Chiba S, Ikeda R, Kurokawa MS, Yoshikawa H, Takeno M, Nagafuchi H, et al: Anatomical and functional recovery by embryonic stem cell-derived neural tissue of a mouse model of brain damage. **J Neurol Sci 219:**107–117, 2004
11. Chiocca EA: Gene therapy: a primer for neurosurgeons. **Neurosurgery 53:**364–373, 2003
12. Corvino V, Businaro R, Geloso MC, Bigini P, Cavallo V, Pompili E, et al: S100B protein and 4-hydroxynonenal in the spinal cord of wobbler mice. **Neurochem Res 28:**341–345, 2003
13. Ehtesham M, Kabos P, Gutierrez MAR, Chung NG, Griffith TS, Black KL, et al: Induction of glioblastoma apoptosis using neural stem cell-mediated delivery of tumor necrosis factor-related apoptosis-inducing ligand. **Cancer Res 62:**7170–7174, 2002
14. Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, et al: Loss of glutamine synthetase in the human epileptogenic hippocampus: possible mechanism for raised extracellular glutamate in mesial temporal lobe epilepsy. **Lancet 363:** 28–37, 2004
15. Erdo F, Buhrle C, Blunk J, Hoehn M, Xia Y, Fleischmann B, et al: Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. **J Cereb Blood Flow Metab 23:** 780–785, 2003
16. Eriksson C, Ericson C, Gates MA, Wictorin K: Long-term, EGF-stimulated cultures of attached GFAP-positive cells derived from the embryonic mouse lateral ganglionic eminence: in vitro and transplantation studies. **Exp Neurol 164:**184–199, 2000
17. Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, et al: Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. **Development 130:**4217–4227, 2003
18. Germano IM, Fable J, Gultekin SH, Silvers A: Adenovirus/herpes simplex-thymidine kinase/gancyclovir complex: preliminary results of a phase I trial in patients with recurrent malignant gliomas. **J Neurooncol 65:**279–289, 2003
19. Ghazanfari FA, Stewart RR: Characteristics of endothelial cells derived from the blood-brain barrier and of astrocytes in culture. **Brain Res 890:**49–65, 2001
20. Gokhan S, Mehler MF: Basic and clinical neuroscience applications of embryonic stem cells. **Anat Rec 265:**142–156, 2001
21. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, et al: *LMO2*-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. **Science 302:**415–419, 2003
22. Harkany T, Andang M, Kingma HJ, Gorcs TJ, Holmgren CD, Zilberter Y, et al: Region-specific generation of functional neurons from naive embryonic stem cells in adult brain. **J Neurochem 88:** 1229–1239, 2004
23. Hovatta O, Mikkola M, Gertow K, Strömberg AM, Inzunza J, Hreinsson J, et al: A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. **Hum Reprod 18:**1404–1409, 2003
24. Jungling K, Nagler K, Pfrieger FW, Gottmann K: Purification of embryonic stem cell-derived neurons by immunoisolation. **FASEB J 17:**2100–2102, 2003
25. Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, et al: Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. **Nature 418:**50–56, 2002
26. Kyba M, Perlingeiro RCR, Daley GQ: HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. **Cell 109:**29–37, 2002
27. Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF, et al: Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. **Proc Natl Acad Sci USA 97:**6126–6131, 2000
28. McCormack MP, Rabbitts TH: Activation of the T-cell oncogene *LMO2* after gene therapy for X-linked severe combined immunodeficiency. **N Engl J Med 350:**913–922, 2004
29. McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, et al: Transplanted embryonic stem cells survive, differentiate, and promote recovery in injured rat spinal cord. **Nat Med 5:** 1410–1412, 1999
30. Park S, Kim EY, Ghil GS, Joo WS, Wang KC, Kim YS, et al: Genetically modified human embryonic stem cells relieve symptomatic motor behavior in a rat model of Parkinson's disease. **Neurosci Lett 353:**91–94, 2003
31. Paxinos G, Franklin KBJ: **The Mouse Brain in Stereotaxic Coordinates, ed 2.** San Diego, CA: Academic Press, 2001
32. Rainov NG, Ren H: Clinical trials with retrovirus mediated gene therapy—what have we learned? **J Neurooncol 65:**227–236, 2003

33. Ransom BR, Chang-Ling T: Astrocytes, in Winn HR (ed): **Youmans Neurological Surgery, ed 5.** Philadelphia: Saunders, 2003, Vol 1, pp 97–116
34. Rathjen J, Haines BP, Hudson KM, Nesci A, Dunn S, Rathjen PD: Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neuroectoderm population. **Development 129:**2649–2661, 2002
35. Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A: Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. **Stem Cells 21:**546–556, 2003
36. Ridet JL, Sarkis C, Serguera C, Zennou V, Charneau P, Mallet J: Transplantation of human adult astrocytes: efficiency and safety requirements for an autologous gene therapy. **J Neurosci Res 72:** 704–708, 2003
37. Rosler ES, Fisk GJ, Ares X, Irving J, Miura T, Rao MS, et al: Long-term culture of human embryonic stem cells in feeder-free conditions. **Dev Dyn 229:**259–274, 2004
38. Scheffler B, Schmandt T, Schroder W, Steinfarz B, Husseini L, Wellmer J, et al: Functional network integration of embryonic stem cell-derived astrocytes in hippocampal slice cultures. **Development 130:**5533–5541, 2003
39. Selkirk SM, Greenberg SJ, Plunkett RJ, Barone TA, Lis A, Spence PO: Syngeneic central nervous system transplantation of genetically transduced mature, adult astrocytes. **Gene Ther 9:**432–443, 2002
40. Sylvester KG, Longaker MT: Stem cells: review and update. **Arch Surg 139:**93–99, 2004
41. Tortarolo M, Crossthwaite AJ, Conforti L, Spencer JP, Williams RJ, Bendotti C, et al: Expression of SOD1 G93A or wild-type SOD1 in primary cultures of astrocytes down-regulates the glutamate transporter GLT-1: lack of involvement of oxidative stress. **J Neurochem 88:**481–493, 2004
42. Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, et al: Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. **Rheumatology 42:**162–165, 2003
43. Yang P, Hernandez MR: Purification of astrocytes from adult human optic nerve heads by immunopanning. **Brain Res Brain Res Protoc 12:**67–76, 2003
44. Ye P, Bagnell R, D'Ercole AJ: Mouse NG2$^+$ oligodendrocyte precursors express mRNA for proteolipid protein but not its DM-20 variant: a study of laser microdissection-captured NG2$^+$ cells. **J Neurosci 23:**4401–4405, 2003

Manuscript received June 15, 2004.
Accepted in final form March 18, 2005.

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