Multi-germ layer lineage central nervous system repair: nerve and vascular cell generation by embryonic stem cells transplanted in the injured brain

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Object. To restore proper function to a damaged central nervous system (CNS) through transplantation, it is necessary to replace both neural and nonneural elements that arise from different germ layers in the embryo. Mounting evidence indicates the importance of signals related to vasculogenesis in governing neural proliferation and differentiation in early CNS development. Here, the authors examined whether embryonic stem cell (ESC)–derived progenitors can selectively generate both neural and endothelial cells after transplantation in the damaged CNS.

Methods. Injections of 20 nmol N-methyl-d-aspartate created a unilateral striatal injury in 7-day-old rats. One week postinjury, murine ESCs, neural-induced with retinoic acid, were transplanted into the injured striatum. Histological staining, laser confocal microscopy, and transmission electron microscopy of grafted ESCs were performed 1 week posttransplantation.

Conclusions. Transplanted ESCs differentiated into neural cells, which segregated into multiple pools and formed neurons that conformed to host cytoarchitecture. The ESCs also generated endothelial cells, which integrated with host cells to form chimeric vasculature. The combination of ESC pluripotentiality and multiple germ layer differentiation provides a new conceptual framework for CNS repair.

Key Words • differentiation • endothelial cell • neural element • pluripotent cell • progenitor cell • regeneration • rat

Replacing and restoring neural elements is the primary focus of therapies that aim to repair the injured CNS.1,2 Although the CNS is capable of incremental spontaneous regeneration, optimal repair requires reconstitution of neural as well as nonneural elements.3,6-11 The vascular system delivers nutrients, removes waste, and may play a crucial role in the delivery of signals that regulate organ development.12,13 Therefore, the repair of blood vessels is considered central to the repair of other organs.14,15 Additionally, mounting evidence indicates that signals from the microvasculature help regulate neurogenesis and neural differentiation.16 Thus, simultaneous replacement of nonneural—that is, endothelial as well as neural cells—could be important for CNS repair.

Abbreviations used in this paper: CNPase = 2',3'-cyclic nucleotide 3'-phosphohydrolase; CNS = central nervous system; DAB = 3,3'-diaminobenzidine; EEM = extraembryonic marker; EGFP = enhanced green fluorescent protein; EMA = epithelial membrane antigen; GABA = γ-aminobutyric acid; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; Ig = immunoglobulin; NMDA = N-methyl-d-aspartate; PND = postnatal day; SSEA = stage-specific embryonic antigen; TH = tyrosine hydroxylase; VEGF = vascular endothelial growth factor; 5-HT = 5-hydroxytryptamine.

Data from studies focused on stem cell plasticity—for example, neural derivation of hematopoietic stem cells and, conversely, hematopoietic derivation from neural stem cells—allow for the possibility that dual replacement may be achievable.13,14,15 Note, however, that the environmental signals that harness stem cell plasticity have remained elusive. Although adult mouse bone marrow–derived hematopoietic progenitor cells express neural genes,16 environmental signals provide a critical factor in brain repair given that grafted previously derived hematopoietic stem cells from the aorta-gonads-mesonephros region of embryonic mice are unable to myelinate shiverer mouse brain after transplantation.17 Other nonneural cell precursors, including muscle-derived myogenic ones, have been proposed for neural cell generation after transplantation.18 Importantly, transplanted myogenic precursors preferentially organized along host blood vessels, indicating their possible importance in microvascular signaling and gene delivery systems, but were unable to reconstitute chimeric vasculature or derive neural cells.19

Given their pluripotency, ESCs are well suited for an examination of these complex issues. These cells can generate the three main types of nervous system cells: neurons, oligodendrocytes, and astrocytes, however, because of the
Transplanted embryonic stem cells

potential to form teratomas (healthy tissues in abnormal locations), there have been doubts about harvesting this capacity for tripotential development. In this report, we investigated whether ESCs can effectively serve as early progenitors of both neural and nonneural cells in the injured immature murine brain.

Materials and Methods

Cell Culture

Both B5 (strain 129SvJ; A. Nagy, University of Toronto, ON, Canada) and K/D3 (strain 129SvJ; D.I. Gottlieb, Washington University, St. Louis, MO) male murine ESC lines were maintained at low passage (< 15 passages in our lab) with normal karyotypes. To identify transplanted B5 ESCs, continuous EGFP expression was optimized in the clonal B5 ESC line by continuous inclusion of G418 (gentamicin 500 μg/ml) in the culture medium during passage of undifferentiated ESCs prior to neural induction and transplantation. In a separate set of experiments, the K/D3 cell line was used. Here, undifferentiated K/D3 ESCs were cotransfected by electroporation with a plasmid carrying the EGFP gene driven by the cytomegalovirus promoter (pEGFP N1; Clonetech, Mountain View, CA) and the plasmid PGK-neo to provide selection. The G418-resistant clones were selected, pooled, and partially purified through cell sorting to create a polyclonal line expressing GFP. Note that expression of GFP was stable after multiple passages in both ESC lines.

Next, both ESC lines were subjected to the 4–/−/+ retinoic acid protocol and induced to differentiate by withdrawing leukemia inhibitory factor (CHEMICON International, Inc., Temecula, CA) and passage into ESC induction medium. As an additional method of identifying transplanted ESCs, which express GFP, the cells were prelabeled with Hoechst 33342 nuclear label. Embryoid bodies derived from B5 4–/−/+ ESCs and killed by repeated freezing were processed for transplantation and used as a control.

Excitotoxic Injury

To produce well-demarcated lesions in the striatum, we used an NMDA-induced excitotoxic lesion. This model provides a reproducible injury to begin to evaluate the factors that regulate ESC differentiation after transplantation. Thirty-five 7-day-old male and female Sprague–Dawley rat pups were anesthetized using 5% halothane/10% CO2 in isopentane while still frozen. Sections of 4–/−/+ embryoid bodies were also prepared, and all immunohistochemical analysis was completed as previously described with primary antibodies (dilutions given) against nestin 130 (rabbit polyclonal antibody, 1:1000; R.D.G. McKay, Bethesda, MD), NeuN (mouse IgG, 1:300; CHEMICON International, Inc.), MAP-2 (mouse IgG, 1:40; CHEMICON International, Inc.), glialfate (mouse IgG, 1:2000; DiaSorin), TH (mouse IgG, 1:200; CHEMICON International, Inc.), 5-HT (rabbit polyclonal antibody, 1:500; DiaSorin, Inc., Stillwater, MN), GABA (rabbit polyclonal antibody, 1:1000; CHEMICON International, Inc.), platelet-derived growth factor–R7 (rabbit polyclonal antibody, 1:200; C.H. Heldin, Ludwig Institute for Cancer Research Uppsala, Sweden), Rip (mouse IgG, 1:50; Developmental Studies Hybridomas Bank), SSEA1 (mouse IgM, 1:50; Developmental Studies Hybridomas Bank, University of Iowa, Iowa City, IA), SMA 91 mouse IgG (2,3-cyclic nolipid 3-phosphodiesterase, 1:500; Sternberger Monoclonals, Inc.), GFAP (rabbit polyclonal antibody, 1:4; DiaSorin, Inc.), anti–mouse CD-3 (rat IgG, 1:20; BD Pharmingen, San Diego, CA), anti–mouse Flk-1 (rat IgG, 1:200; BD Pharmingen), GLUT-1 (rabbit polyclonal antibody, 1:750; CHEMICON International, Inc.), anti–mouse EEM-1 (hamster IgG, 1:10; D.I. Gottlieb, Washington University School of Medicine, St. Louis, MO), SSEA-1 (mouse IgM, 1:50; Developmental Studies Hybridomas Bank), SSEA/LeX (1:200; S. Temple, Albany Medical College, Albany, NY), anti–mouse EMA (rat IgG hybridoma, 1:4; Developmental Studies Hybridomas Bank), anti–mouse M2 (rat IgM, 1:4; C. Lagenaur, University of Pittsburgh, Pittsburgh, PA), anti–mouse Thy-1.2 (rat IgM, 1:1000; Serotec), anti-GFP (rabbit IgG/Alexa 488, 1:500; Molecular Probes, Carlsbad, CA), and anti-GFP (mouse 3E6, 1:1000; Molecular Probes); and secondary antibodies (all from CHEMICON International, Inc.) against goat-derived CY-3 (1:300), goat-derived CY-5 (1:300), goat-derived Alexa 488 (1:300), goat-derived biotinylated anti–hamster antibody (1:200), and goat-derived CY-3 avidin (1:300). Primary and secondary control slides were included with each stain series.

Cell-type counts in embryoid bodies were quantified based on immunoreactivity to phenotypic markers. Immunopositive cells were reported as percentages of the total number of nuclei counterstained with Hoechst 33342 within each embryoid body. For in vivo evaluation, four ESC-transplanted and sham-operated control tissue sections per animal (four–seven animals) were scored for immunoreactivity within their observed spatial parameter (Table 1). The rating scale for immunoreactivity of transplanted cells (identified by GFP or mouse marker expression) was scored as follows: +, weak expression; ++, moderate expression; ++++, strong expression; and ++++++, intense expression. Neurite outgrowth expressing GFP was evaluated using a similar rating scale slightly modified as follows: 0, no process outgrowth; +, a few scattered fibers; ++, several fibers in selected areas of a region; and ++++, several fibers throughout a region; ++++++, dense fibers throughout a region (Table 2).

Transmission Electron Microscopy

On PND 22, eight rats (four transplanted with B5 cells and four with sham vehicle) were perfused with paraformaldehyde with 0.25% glutaraldehyde to facilitate postembedding immunolabeling of GFP. A vibratome was used to cut 50-μm brain tissue sections, which were washed 10 times for 5 minutes each time in Tris/phosphate buffer and then incubated for 30 minutes in 10% NGS. Tissue sections were washed again thrice for 5 minutes each time and then incubated in primary antibody overnight at 4˚C. Next, the sections were washed thrice for 5 minutes each time and incubated for 30
Prelabeling

A thorough transmission electron microscopy methods.

We carefully examined the cerebral tissue in all the rats for teratomas and found no morphological, immunocytochemical, or ultrastructural evidence of abnormal non-CNS tissue (data not shown).

* Posttransplantation ESC Survival

We transplanted the ESCs into rat pups that had been injured 1 week earlier with an intratracheal injection of NMDA. One week postinjury, we consistently observed a selective loss of neurons in the injured tissue of all 13 NMDA-injured animals. On PND 22, rats were assessed for transplanted ESC survival. Tissue sections displayed robust cellular survival including extensive neural process projections. We identified the transplanted cells (Fig. 2E–P) by using a combination of three fluorescent marker systems: Hoechst nuclear pretransplantation labeling, molecular GFP expression, and anti–mouse (ESC)-specific antibodies (that is, EMA, Thy1.2, and M2).

The ESCs were identified the ESCs by DAB precipitation for molecular anti–mouse marker identification of transplanted cells, although or ultrastructural evidence of abnormal non-CNS tissue (data not shown).

**Survival and Chimeric Microvasculature of ESC-Derived Vascular Cells**

We examined representative thin plastic-embedded and

dermal and endothelial lineage cells. Fewer than 5% of the cells immunostained for platelet endothelial cell adhesion molecule (CD-31; Fig. 1E). Another group of nonneural cells, covered with microprocesses, formed a layer around the embryoid bodies. They labeled with anti–EEM-1 (Fig. 1F) and resembled the extraembryonic visceral endoderm layer. Thus, the 4−/4+ embryoid bodies contained neural precursors and at least two types of nonneural precursors, including an Flk-1-immunoreactive hematopoietic precursor.

**Spatial distribution of immunoactive transplanted ESCs**

<table>
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<tr>
<th>Primary Antibody</th>
<th>Average Score</th>
<th>Location</th>
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<td>NeuN</td>
<td>++++</td>
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</tr>
<tr>
<td>nestin</td>
<td>++</td>
<td>throughout graft</td>
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<tr>
<td>Rip</td>
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<td>+</td>
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<tr>
<td>GFAP</td>
<td>+</td>
<td>crescent-aligned cell groups around vessels</td>
</tr>
<tr>
<td>NG2</td>
<td>+</td>
<td>crescent-aligned cell groups around vessels</td>
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* Primary antibodies are scored based on immunoreactivity of transplanted cells identified (double-labeled) by either GFP expression or anti–mouse markers (EMA, M2, or Thy 1.2). Rating scale: + = weak expression; ++ = moderate expression; +++ = strong expression; ++++ = intense expression.

**Spatial distribution of transplanted ESC-derived process outgrowth**

<table>
<thead>
<tr>
<th>No.</th>
<th>Corpus Callosum</th>
<th>Striasome</th>
<th>Ant Commissure</th>
<th>Win/In Graft</th>
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<tbody>
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<td>+</td>
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<td>9</td>
<td>+</td>
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* Green fluorescent protein expressing processes were scored on the following rating scale: 0 = no axon outgrowth; + = few scattered fibers; ++ = several fibers in selected areas of a region; +++ = several fibers throughout a region; ++++ = dense fibers throughout a region.

**Results**

**Endothelial Precursors in 4−/4+ Embryoid Bodies**

By exposing ESCs to retinoic acid during the last 4 days of an 8-day period, we derived a population of murine and a small percentage of hematopoietic/endothelial lineage cells. Fewer than 5% of the cells were positive for nestin (neural precursor), and 6% were positive for NeuN (neurons). In the current study, a large percentage of cells in the embryoid bodies derived from B3 and KD3-GFP ESCs were similarly positive for nestin 130 (Fig. 1C), which is expressed by undifferentiated ESCs and immature ectoderm. Two to five percent of the cells within the embryoid bodies immunolabeled with antibodies against Flk-1 (Fig. 1D), an antigen for endothelial cell-specific VEGF receptor. The Flk-1-immunopositive cells predominate when embryoid bodies reach the 3- to 3.5-day stage and lineage selection is limited by retinoic acid.

**Table 1**

Spatial distribution of immunoactive transplanted ESCs

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**Table 2**

Spatial distribution of transplanted ESC-derived process outgrowth
Transplanted embryonic stem cells

frozen sections to determine whether transplanted 4−/4+ ESCs generate vascular endothelial cells. We used anti–GLUT-1 immunoreactivity to label endothelial cells, which revealed numerous capillaries at the transplantation site. Immunomagnetic cell sorting analysis revealed cells with thin nuclear profiles and darkened chromatin and basal lamina (Fig. 3A). Confocal microscopy demonstrated colocalization of anti–GLUT-1, anti-GFP, anti–mouse (ESC)-specific antibody, and Hoechst 33342 epifluorescence in endothelial cells consisting of microvessels in and around the transplantation site. Colocalization of GFP expression (without antibody amplification) with GLUT-1−positive immunoreactivity in frozen sections confirmed, without the potential confounder of cross-species immunolabelling, that the endothelial cells were derived from transplanted ESCs (Table 1). Additional evidence of an endothelial identity for these ESC-derived cells included elongated morphological features, thin nuclear profiles, and lumens adjacent to cell surfaces (Fig. 3B–E). Colocalization of GFP and GLUT-1 was also observed at a distance of 1.5 to 2 mm from the transplant epicenter. Confocal examination revealed that the ESC-derived endothelial cells had aligned with host endothelial cells (Fig. 3G–K) to form chimeric microvasculature.

Association Between ESC–Derived Neural Progenitor Cells and Host Microvasculature

A separate subgroup of ESC-derived cells expressing GFP appeared to be using host microvasculature for migration. They formed crescent-shaped bodies around the capillaries and were consistently seen along capillaries emanating from the transplant site to all regions of the host striatum (Fig. 4A and B). We confirmed their origin by detecting spontaneous and amplified GFP expression in frozen sections and anti-GFP expression in thin plastic sections (Fig. 4D and E). The cells did not label with the endothelial cell marker GLUT-1 or with neural markers (NeuN, Rip, CNPase), but they were NG2-positive (Table 1), exhibited a high nuclear/cytoplasmic ratio, and had a few short processes. This profile resembles that of precursors or earlier neural progenitors. Immediately around capillaries, we also found few ESC-derived cells that expressed GFAP and whose ultrastructure resembled that of pericytes and astrocytes, which normally associate with capillaries (Fig. 4D and E).

Neural Differentiation and Integration of ESCs in Injured Immature Murine Brain

Most of the transplanted ESCs differentiated into cells whose cytoarchitectural, morphological, and immunocytochemical features resembled those of mature neurons (Fig. 5A–E). The new neurons immunolabeled with neuronal label anti–NeuN (Table 1) and exhibited bipolar specialization with spatially distinct immunolabeling of presumptive axons (SMI 31 and SMI 311) and dendrites (MAP-2). We identified dendrites, axons, and synapses in ultrastructural studies (Fig. 5M). The cells also expressed immunoreactivity for GABAergic, glutamatergic (anti-glutamate), serotonergic (anti–5-HT), and dopaminergic (anti-TH) phenotypes, were located at or within 2 mm of the engraftment site, and were observed across multiple single sections (nine of nine animals) labeled per marker (Fig. 5F–I). Results of confocal microscopy revealed axons extending for long distances, and punctate labeling for presynaptic proteins (synaptophysin) indicated putative synaptic boutons (Fig. 5J–L). Ultrastructural evidence demonstrated dense-cored vesicles grouped at the soma periphery, which is consistent with early neuronal development and their requirement for synapse development (Fig. 5N).
A small proportion of transplanted ESCs differentiated into mature glial phenotypes. A large portion resembled astrocyte-like cells, judging from their GFAP immunoreactivity and cytoarchitectural features (Table 1). Nevertheless, immunohistochemical analysis revealed few cells expressing immunomarkers commonly expressed in mature oligodendrocytes (anti-Rip and anti-CNPase). Immuno-transmission electron microscopy confirmed the presence of ESC-derived astrocytes and immature oligodendrocytes (Fig. 5O and P). A small number of mitotically active, GFP-expressing cells with ultrastructural features consistent with immature ESC-derived cells (for example, Ki-67–positivity...
Transplanted embryonic stem cells

Processes Extending from ESCs Conform to Host Cytoarchitecture

The ESC-derived cells that expressed GFP extended processes locally and over long distances (Table 2). Note that

and a pattern of nuclear chromatin Hoechst staining consistent with active cellular division; data not shown) were observed (Fig. 5Q). These dividing subpopulations were consistent with neural (antinestin 130) and oligodendroglial precursors (anti-NG2).  

Fig. 3. Embryonic stem cell–derived vascular cells survive and create chimeric microvessels. A: Transmission electron micrograph of the basal lamina (arrowheads) demonstrating a GFP-labeled endothelial cell derived from a transplanted ESC. Arrows indicate DAB precipitate. Images of multichannel epifluorescence from the same field illustrating ESC-derived endothelial cells with the concave side on the lumen edge (arrow); thin, elongated ESC nuclei prelabeled with Hoechst 33342 (B); anti–GFP-labeled (green) semicircular and elongated cells (C); and anti–GLUT-1 localization (red, D). E: Merged image of C and D. Vascular lumens (arrows) are seen throughout the primary graft site (GFP = green). Images depicting ESC-derived immature vessel that contributes to existing microvasculature: vascular identification by prelabeling with Hoechst 33342 (blue, G) or labeling with anti-GFP (H) or anti–GLUT-1 (red, I). J: Merged image of H and I. Yellow star indicates vessel-like structure. K: Schematic of the chimeric vessel in panels G to J. Endothelial cells derived from ESCs are shaded dark to represent the identifying fluorescent label. Bars = 1 μm (A), 5 μm (B–K).
the projection patterns conformed closely to host cytoarchitecture. For example, within the striatum, the ESC-derived neuronal axons primarily extended within the spatial confines of the endogenous striatal striasomes (bundles of myelinated axons). Antibodies to myelin-associated proteins (anti-Rip and anti-CNPase) revealed myelin-figure cross-sections of the chimeric striasomes (Fig. 6A–E). Confocal microscopy of double immunolabeling and electron microscopy showed most of the GFP-expressing processes in the striasomes as axons based on expression of immunoreactivity to neurofilaments.

Few ESC neuronal cell bodies were located in white matter tracts, but the GFP-expressing processes of these cells grew parallel to endogenous while matter tracts, extending for long distances along the corpus callosum (projected > 4 mm in eight of nine animals) and the anterior commissure (projected > 3 mm in four of nine animals; Fig. 6F and G). Thus, ESCs have the potential to migrate, rapidly extend long-distance axonal projections, and repair areas distant from the transplant site.

Discussion

The major contribution of the work presented here is that bi-germ layer survival, differentiation, and anatomical integration is achieved by transplantation of ESC progenitor cells in an anatomically appropriate pattern. Embryonic stem cells transplanted into the injured CNS can generate anatomically appropriate cells derived from two embryonic germ layers: ectoderm (neural) and endoderm (vascular). Transplanted ESCs gave rise to endothelial cells, thus contributing to chimeric microvasculature while simultaneously replacing neural cells. Interestingly, the two germ layer products maintained spatially distinct associations. We also found that surviving ESCs were multipotent, segregated into distinct progenitor pools, and capable of migration. The neural cells differentiated primarily into neurons that exhibited phenotypes appropriate for striatal neurons, including GABAergic, glutamatergic, serotonergic, and dopaminergic phenotypes. Their long processes conformed to host cytoarchitecture and exhibited appropriate immunocytochemical and ultrastructural properties, including synapses and dense-core vesicles. Interestingly, prominent long-distance fiber outgrowth was demonstrated not only within the transplantation injury site, including myelinated striatal striasomes, but also in white matter tracts away from the injured area.

Multilineage Contribution to CNS Organogenesis

Current concepts in CNS regeneration include neural cell replacement and creation of an environment conducive to axonal growth and targeting. Note, however, that neo-

Fig. 4. Neural progenitor cells derived from ESCs associate with host microvasculature. Away from the primary transplant site, GFP-expressing ESCs (green) migrate along vascular structures and form crescent-shaped groups. Yellow star indicates the vessel lumen. Arrows point to ESCs in phase-contrast image (A), anti-GFP epifluorescence image (B), and a schematic (C). Electron microscopic images demonstrating ESC-derived pericytes (D) and astrocytes (E) with abutting foot processes. Arrows represent GFP label; arrowhead indicates nucleus. Bar = 1 μm.
vascularization of injured tissue ultimately must be considered. Growing evidence of a link between neovascular signaling and neurogenesis offers a unique genetic target for therapeutic reconstitution. Dual differentiation of ESC-derived cells into vascular endothelium offers a unique approach for genetic therapies.

In this study, we examined the potential of ESCs transplanted into the NMDA-injured CNS, with resultant neural integration and neurotransmitter phenotypes. Confocal epifluorescence images demonstrating an ESC-derived neuron (anti-GFP, green, J) extending within the host striatum. Synaptophysin (anti-Synap, red, K) immunoreactivity is indicated by yellow arrows at the somata and along neurites, reflecting presumptive synaptic connections (merged image, L). Ultrastructural studies showing dendrites (arrow in M indicates the GFP label) and dense-core vesicles (N, inset, red arrows) grouped at the periphery of the ESC-derived neurons. Transmission electron micrographs revealing transplanted ESC-derived glia: astrocytes (O, arrow indicates GFP label) and a medium-sized oligodendrocyte (P, arrow indicates GFP label). Q: Electron micrograph depicting some transplanted ESCs, which remain mitotically active (bold yellow arrow indicates dividing chromatin in the nucleus; small arrow indicates GFP-related DAB precipitate in the cytoplasm) at 1 week posttransplantation. Bars = 10 (D, F–I, and L), 2 (E and M–Q), and 0.25 μm (inset).

Fig. 5. Neural differentiation and integration of ESCs in the injured immature rat brain. Transplanted ESC-derived progenitors differentiate into neurons with multiple neurotransmitter phenotypes. Epifluorescence demonstrates Hoechst 33342 prelabel (blue, A), anti-GFP (green, B), anti-NeuN (red, C), and merged images (D). E: Electron micrograph of a transplanted ESC-derived neuron displaying a characteristic pale nuclear profile and a clear nucleolus. Arrow represents the GFP label. Triple-channel epifluorescence images showing that transplanted ESC-derived neural cells are immunoreactive for GABA (F), glutamate ([Glu], G), 5-HT ([H], H), and TH (I, each neurotransmitter marker is shown in red, the Hoechst nuclear label in blue, and the GFP in green). Confocal epifluorescence images demonstrating an ESC-derived neuron (anti-GFP, green, J) extending within the host striatum. Synaptophysin ([Synap], red, K) immunoreactivity is indicated by yellow arrows at the somata (white star; Hoechst 33342 nuclear labeling not shown) and along neurites, reflecting presumptive synaptic connections (merged image, L). Ultrastructural studies showing dendrites (arrow in M indicates the GFP label) and dense-core vesicles (N, inset, red arrows) grouped at the periphery of the ESC-derived neurons. Transmission electron micrographs revealing transplanted ESC-derived glia: astrocytes (O, arrow indicates GFP label) and a medium-sized oligodendrocyte (P, arrow indicates GFP label). Q: Electron micrograph depicting some transplanted ESCs, which remain mitotically active (bold yellow arrow indicates dividing chromatin in the nucleus; small arrow indicates GFP-related DAB precipitate in the cytoplasm) at 1 week posttransplantation. Bars = 10 (D, F–I, and L), 2 (E and M–Q), and 0.25 μm (inset).
and vascular injury, to selectively differentiate into neural and vascular cells. We provided immunocytochemical, morphological, and ultrastructural evidence for the derivation of endothelial cells from hematopoietic precursors originating in the embryoid body and from ESC-derived progenitors transplanted in the damaged CNS as well as for the integration of these cells into chimeric microvessels. In addition, some ESCs immediately around microvessels appeared to have differentiated into pericytes. These glial-lineage cells associate with blood vessels, express NG2 immunoreactivity, have characteristic ultrastructure, and differentiate from transplanted embryonic striatal and hippocampal neural stem cells. It is unlikely that the GFP expression in endothelial cells is a false-positive indicator of ESC-derived cells for several reasons: GFP expression is observed without antibody amplification, endothelial cells exhibit high levels and appropriate subcellular expression of GFP, GFP expression is selective with adjacent endothelial cells not expressing GFP, no endothelial GFP expression is observed in control animals in which dead GFP-expressing ESCs or sham media were transplanted, and similar endothelial identities were observed with the other markers.

After injury, endothelial cells differentiate, at least partially in response to vasculogenic cues. Also, recent evidence indicates that endothelial cells and neovascularization provide important signals for initiating embryonic development of the liver and pancreas. Therefore, endothelial signals might also be critical for initiating and/or maintaining an injury-appropriate regeneration program in the CNS. Furthermore, VEGF concomitantly stimulates neurogenesis when it promotes vasculogenesis in the adult rat brain. Our observation that crescent-shaped groups of ESC-derived neural progenitors frequently surround vessels emanating from the graft site was not unexpected, given that neural and vascular progenitors respond to the same mitogenic factors, including basic fibroblast growth factor, epithelial growth factor, platelet-derived growth factor, and now VEGF. These factors also participate in the signaling of migration. After detecting clusters of dividing cells around the vasculature in the hippocampus in mature rodents, Palmer, et al., posited that neurogenesis occurs within an angiogenic niche. Louissaint, et al., found a correlation between angiogenesis and recruitment of new neural cells in the adult songbird and concluded that vasculature might enhance cues to neurogenic development.

In this study, we did not complete viral lineage analysis tracking within the transplant. Note, however, that 2 to 5%
Transplanted embryonic stem cells

of the embryoid body cells were immunopositive for Flk-1, a specific vascular progenitor marker. Moreover, the embryoid bodies contained a similar percentage of cells that were CD-31–positive (PECAM-1, which does not distinguish precursor cells from immature or mature endothelial cells). The embryoid bodies lacked blood vessels. Thus, the most likely ancestor of the ESC-derived endothelial cells was the Flk-1–positive endothelial progenitor. Nevertheless, we cannot exclude the possibility that these cells arose from a precursor common to the endodermal and ectodermal lineages or transdifferentiated from neural precursors.2

Based on recent evidence, one can infer that spontaneous fusion may play a role in cellular reprogramming and differentiation.1,0,73 Perhaps some of the endothelial cell labeling results from the fusion of transplanted cells and vascular cells. Although this process occurs at a very low rate following systemic vascular transplantation, such fusion from direct transplantation has not been observed. Nevertheless, the magnitude of labeled endothelial cells in the present study exceeded that previously reported from cell fusion by orders of magnitude, making cell–cell fusion an unlikely candidate to explain these results. Even so, we cannot exclude a small contribution by cell fusion.

Dual germ layer transplantation may have important implications regarding the treatment of CNS disease. First, there is a clear link between vasculogenesis and organogenesis, and regulating this process may require control of both neural and vascular elements. Second, angiogenesis is felt to be a limiting factor following CNS ischemia. Promotion of vasculogenesis can enhance recovery and repair in models of stroke.19,60 In the present study, preliminary, qualitative observations of ESC-transplanted rats revealed that eight of 13 rats had reduced excitotoxic-induced damage to the striatum (reduction of injury-induced ventricular dilation and increased cross-sectional striatal area scores) compared with seven sham-operated controls (unpublished observations). Furthermore, genetic delivery via endothelial cells is an important therapeutic target that may be amenable to dual germ layer transplantation.

Neural Differentiation and Integration of Transplanted ESCs

Identification of transplanted ESCs based on differentiation and cell location can provide clues regarding the general responsive behavior of stem cells to their microenvironment, providing implications for both regeneration and cell replacement strategies. The large-scale neuronal and vascular cell differentiation within the transplantation injury site documented in the present study indicates that the injured immature CNS permits regeneration of both neural and vascular components. Progenitor cell differentiation within distinct populations, that is, NG2-positive cells surrounding host blood vessels, indicates that migratory patterns of transplanted neural stem cells represent the possibility that a preference for migration along host blood vessels may exist. Segregation of transplanted human neural progenitors has also been observed in the intact immature monkey CNS,54 indicating that different progenitor pools might utilize distinct migration routes. The NG2-positive, crescent-shaped clusters around blood vessels reported here with GFP-transfected cell expression and ultrastructural identification confirms earlier observations of Lundberg, et al.,44 who observed 3H-thymidine–labeled transplanted cells localized around blood vessels comparable to pericytes. Furthermore, Englund, et al.,26 asserted that these populations may represent an undifferentiated pool of cells because of their absence at long-term survival points. These migratory patterns were found only within injured striatal parenchyma, further indicating the importance of CNS injury signaling. Further elucidation of transplanted neural stem cell segregation patterns may have important implications regarding CNS organogenesis and repair.

Our documentation of ESCs transplanted into the injured immature brain expressing patterns of neuronal and glial differentiation extends similar results following mouse and human ESC transplantation in other CNS injury models.9,10, 14,20,27,35,36,38,41,49,57,70,74 In the current study ESC-derived endothelial cells formed chimeric microvasculature and pericyte-like cells arranged themselves around the vasculature. Concomitantly, we found that neuronal differentiation occurred with the expression of multiple phenotypes typically found in the immature rat striatum. Additionally, neuronal processes extended long distances, demonstrating an outgrowth pattern in the injured striatum similar to transplanted ESC axons conforming to intact striatal development.10 while also expressing myelin and presynaptic markers. Chimeric reconstruction, immunofluorescent marker expression, and our ultrastructural data, which provide an additional level of confirmatory analysis unavailable in most previous studies of neural progenitor transplantation, indicate that ESCs transplanted in the injured immature rat brain may be suitable for further studies of functional integration26,59 and behavioral improvement.10,35,36,46

Conclusions

The ability of transplanted ESCs to differentiate into cells that normally derive from multiple embryonic germ layers could be advantageous in regenerating damaged CNS. Although it was known that ESCs could generate multiple germ layers and theoretically differentiate into all cell types, data from the current in vivo study reveals three novel concepts. First, transplanted ESCs can differentiate into multiple germ layers for the purpose of simultaneously replacing neural and vascular elements. Second, endothelial cells derived from transplanted ESCs integrate appropriately with host cells to form a chimeric microvasculature. Third, transplanted neural stem cells exhibit migration patterns in association with host vasculature. Because repairing the injured CNS will ultimately require multistage interventions to replace both neural and nonneural elements, these findings underscore the therapeutic potential of ESC transplantation for treating the injured CNS.

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