Embryonic Stem Cell–Derived Glial Precursors: A Source of Myelinating Transplants

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Self-renewing, totipotent embryonic stem (ES) cells may provide a virtually unlimited donor source for transplantation. A protocol that permits the in vitro generation of precursors for oligodendrocytes and astrocytes from ES cells was devised. Transplantation in a rat model of a human myelin disease shows that these ES cell–derived precursors interact with host neurons and efficiently myelinate axons in brain and spinal cord. Thus, ES cells can serve as a valuable source of cell type–specific somatic precursors for neural transplantation.

Embryonic stem cells derived from the inner cell mass of blastocyst-stage embryos are totipotent cells that can differentiate into all tissues and cell types (*1*). Recent discoveries that extend the potential use of ES cells include the isolation of ES cells from embryonic human tissue (*2*) and transplantation in sheep and mice of nuclei from mature tissues into enucleated oocytes, permitting the generation of ES cells from the same individual (*3*). Thus, ES cell technology may be the basis of new cell replacement therapies.

ES cells induced to differentiate in vitro give rise to many cell types including hematopoietic precursors, heart and skeletal muscle, endothelium, and neural cells (*4*). In the central nervous system, proliferation and differentiation of multipotential neural stem cells and glial progenitors can be controlled by defined factors (*5*). Here, we show that applying this knowledge to ES cells permits efficient in vitro generation of precursors for oligodendrocytes and astrocytes. Transplant studies indicate that oligodendrocyte precursors myelinate host axons in a variety of animal models for myelin diseases (*6*), suggesting that these developments in ES cell technology could be useful in the clinic.

To initiate differentiation, ES cells were aggregated to embryoid bodies and plated in a defined medium that favors the survival of ES cell–derived neural precursors (*7, 8*). Cells were then passaged and sequentially propagated through media containing (i) basic fibroblast growth factor (FGF2), (ii) FGF2 and epidermal growth factor (EGF), and (iii) FGF2 and platelet-derived growth factor (PDGF); the latter is a growth factor combination known to promote the proliferation of glial precursor cells (*5*). These conditions yielded an isomorphous population of round to bipolar cells with immunoreactivity to the monoclonal antibody A2B5, which recognizes a membrane epitope typically expressed in glial precursors (Fig. 1A) (*9*). Upon growth factor withdrawal, the cells differentiated into oligodendrocytes and astrocytes (*10*). Four days after withdrawal, 38.3 ± 5.8% (mean ± SEM of three experiments) of this population were immunoreactive to O4, an antibody recognizing oligodendrocyte-specific glycolipids (*11*). Many of the cells showed a multipolar morphology characteristic for oligodendrocytes (Fig. 1B). At the same time, 35.7 ± 6.4% of the cells expressed the astrocytic marker antigen glial fibrillary acidic protein (GFAP) and exhibited a flat morphology typical of cultured astrocytes (Fig. 1C). Prolonged growth factor withdrawal for more than 5 days promoted further oligodendroglial differentiation and expression of myelin proteins such as 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (*12*). Cells growing in FGF2/EGF- and FGF2/PDGF-containing media could be frozen and thawed without losing their potential for oligodendroglial and astrocytic differentiation.

To investigate whether ES cell–derived oligodendrocytes can form myelin in vivo, cells grown in the presence of FGF2 and PDGF were transplanted into the spinal cord of 1-week-old myelin-deficient (md) rats, an animal model for the hereditary human myelin disorder Pelizaeus-Merzbacher disease (PMD) (*13*). Both, PMD patients and md rats carry mutations in the X-linked gene encoding myelin proteolipid protein (*PLP*) (*14*). Affected md rats succumb to their myelin deficiency in the 4th postnatal week and lack PLP-positive myelin. Two weeks after transplantation of ES cell–derived precursors into the dorsal columns of the spinal cord, numerous myelin sheaths were found in six of nine affected md males (*15*). Myelin-forming donor cells were not restricted to the implant site but had spread in both longitudinal and horizontal directions (Fig. 2A). A single injection of 100,000 cells elicited macroscopically visible myelination across a 3-mm seg-

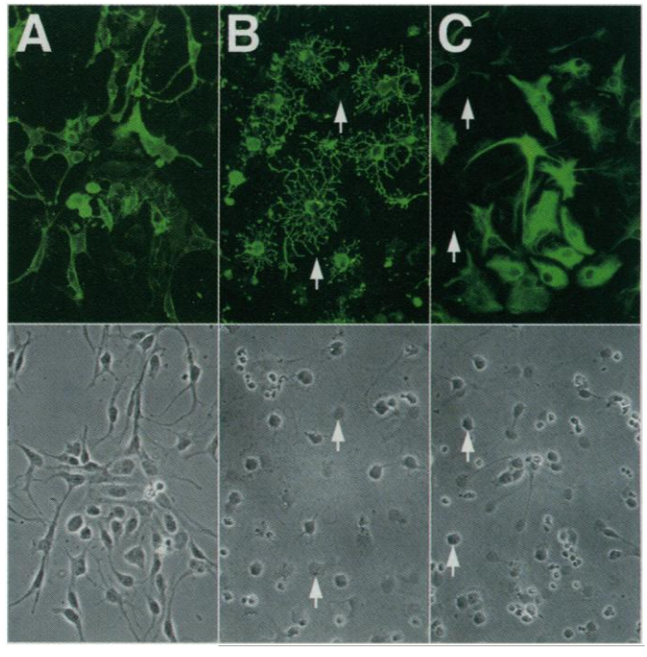

Fig. 1. Morphology and antigen expression of ES cell–derived glial precursors. **(A)** Cells grown in the presence of FGF2 and PDGF show immunoreactivity with the A2B5 antibody. **(B)** Four days after growth factor withdrawal, many cells assumed the typical multipolar morphology of oligodendrocytes and express the oligodendroglial antigen O4. Note the unlabeled cells with a flat, astrocytic phenotype (arrows). **(C)** The same culture contains numerous astrocytes expressing the astrocyte-specific intermediate filament GFAP. Note the unlabeled cells with multipolar oligodendroglial morphology (arrows). Immunofluorescent and phase contrast pictures are shown in the upper and lower panels, respectively.

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ment of the dorsal columns. Donor cells and PLP-positive myelin sheaths were also detected in the spinal cord gray matter and the ventral columns within 2 mm of the injection site. The mouse origin of the PLP and GFAP immunoreactivity was confirmed by in situ hybridization of immunostained sections with a probe to mouse satellite DNA (Fig. 2, B and C). Semithin sections showed numerous myelin sheaths surrounding host axons of different caliber (Fig. 2, D and E). In some cases, the transplanted cells had myelinated more than half of the cross-sectioned area of the dorsal columns. Upon electron microscopic examination, the newly formed myelin sheaths displayed a normal periodicity and variable thickness typical of that seen on remyelination (Fig. 2F). In contrast to host oligodendroglia, grafted oligodendrocytes found within the myelinated areas had a normal ultrastructural appearance. Thus, ES cell–derived glial precursors transplanted into the neonatal rat spinal cord migrate over several millimeters and differentiate into myelinating oligodendrocytes and astrocytes.

Substantial myelin repair requires widespread delivery of the donor cells to the nervous system. Because neural precursors transplanted into the cerebral ventricles can populate large areas of the developing rodent brain (*16, 17*), we performed intraventricular transplants in embryonic day 17 (E17) hosts (*18*). At 3 weeks of age, 9 of 15 affected males (obtained after transplantation of 44 embryos) showed PLP-positive myelin sheaths in a variety of brain regions, including cortex and corpus callosum, anterior commissure, hippocampus, tectum (Fig. 3, A and B), thalamus, and hypothalamus. DNA in situ hybridization confirmed the identity of cells expressing PLP (Fig. 3A) and myelin basic protein (MBP, Fig. 3B) or GFAP (Fig. 3D) (*15*). Although donor cells also incorporated in the brains of unaffected littermates, endogenous expression of PLP and MBP precluded the analysis of donor-derived myelination in these brains. However, double labeling of hybridized cells with cell type–specific markers confirmed that ES cell–derived glial precursors also generate astrocytes and oligodendrocytes in the normal rat brain (Fig. 3C). Thus, ES cell–derived glial precursors transplanted into the ventricle of embryonic hosts migrate and myelinate axons in multiple host brain regions.

Our data show that cell type–specific somatic precursors can be generated from ES cells and used for nervous system repair. Since ES cells can be maintained and expanded in an undifferentiated state (*19*), it is possible to generate virtually unlimited numbers of cells for transplantation. Previous transplant studies involving ES cell–derived neural cells generated without growth factors or with retinoic acid treatment were complicated by the formation of heterogeneous tissues and teratomas (*20*). We noted no signs of tumor growth or non-neural tissue in the transplant recipients. Although long-term survival studies will be necessary to comprehensively address the safety of ES cell transplantation, our observations suggest that the prolonged propagation in growth factors promoting glial pro-

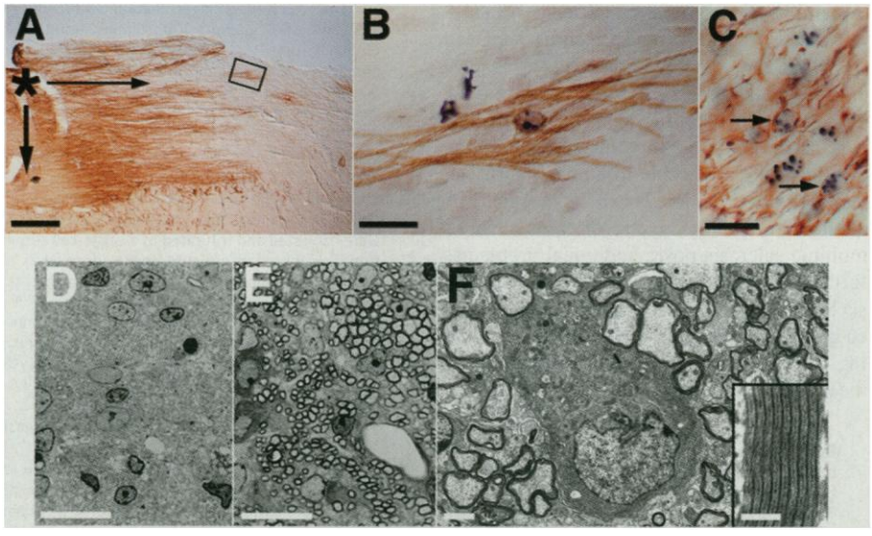

Fig. 2. Differentiation of ES cell–derived glial precursors following transplantation into the spinal cord of myelin-deficient rats. **(A)** Two weeks after transplantation into the dorsal columns of 7-day-old hosts, the transplanted cells generated abundant myelin sheaths. Shown is a sagittal section through the spinal cord, stained with an antibody to PLP. Note that the cells have migrated both longitudinally (thin arrow) and ventrally (thick arrow). The asterisk marks the injection site. **(B)** Detail from (A), showing DNA in situ hybridization with a probe to mouse satellite DNA (black nuclear labeling). **(C)** Double labeling of hybridized cells with an antibody to GFAP also reveals ES cell–derived astrocytes within the myelinated areas (arrows). Toluidine blue–stained semithin sections through the dorsal columns show **(D)** myelin-deficiency in an untreated md rat and **(E)** ES cell–derived myelin formation in an md rat 2 weeks after transplantation. **(F)** Electron microscopic examination of an md rat dorsal column after transplantation of ES cell–derived glial precursors. Shown is a cell with the ultrastructural appearance of an oligodendrocyte contacting numerous myelinated axons. (Inset) High magnification reveals a normal myelin structure. Bars: (A), 200 μm; (B) to (E), 20 μm; (F), 2 μm; (inset), 50 nm.

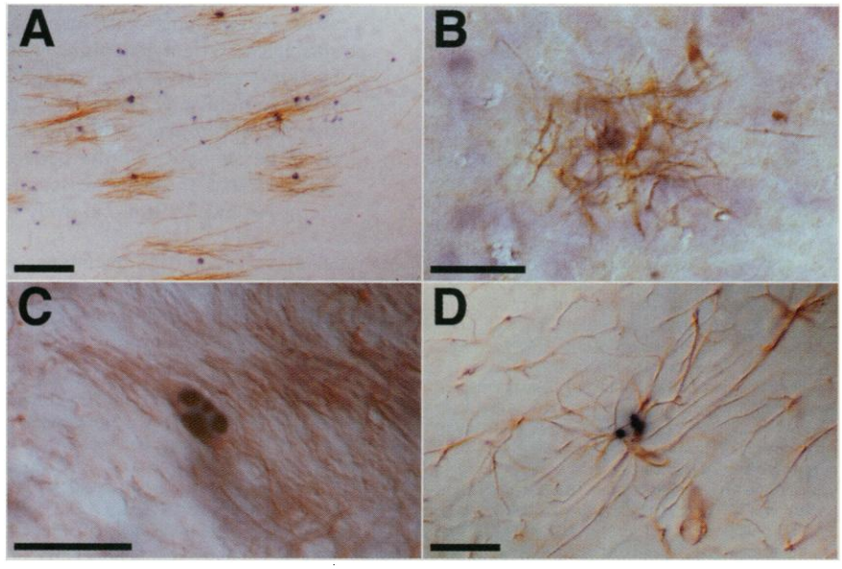

Fig. 3. ES cell–derived glial precursors implanted into the ventricle of E17 rats generate myelin sheaths in multiple brain regions. Shown are representative examples from tectum [**(A** and **B)**; md rat] and hypothalamus [**(C)**; unaffected control animal]. Donor cells are double labeled by DNA in situ hybridization and antibodies to the myelin proteins PLP (A), MBP (B), and CNP (C). **(D)** Donor-derived astrocyte in the md host cortex, showing expression of GFAP. Bars: (A), 100 μm; (B) to (D), 20 μm.

liferation may eliminate undesired cells in the donor cell population. Similar cell culture strategies may be used to extend this approach to other somatic precursor cells.

The availability of human ES cells and the possibility of generating autologous ES cells by nuclear transfer provide exciting perspectives for the treatment of human diseases. The efficient generation of ES cell–derived glial cells and their use in a neonatal myelin disease model indicates that this strategy might eventually be applicable to human neurological disorders. Although cell replacement in adult inflammatory myelin diseases such as multiple sclerosis poses additional problems, further optimization of the donor ES cells such as targeted inactivation of disease-related genes or overexpression of factors promoting cell migration and survival may help to meet these challenges.

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7. ES cells [line J1 (*21*), passage number ≤ 17] were grown on γ-irradiated embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, 0.1 mM 2-mercaptoethanol, nucleosides, nonessential amino acids, and human recombinant leukemia inhibitory factor (LIF, Life Technologies; 1000 units/ml). Cells were passaged once onto gelatin-coated dishes and then aggregated to form embryoid bodies in the absence of LIF. We plated 4-day-old embryoid bodies in tissue culture dishes and propagated them for 5 days in ITSFn medium [DMEM/F12 supplemented with 5 μg/ml insulin, 50 μg/ml transferrin, 30 nM selenium chloride, and 5 μg/ml fibronectin (*8*)]. Cells were then trypsinized, plated in polyornithine-coated dishes (15 μg/ml), and propagated in DMEM/F12 supplemented with insulin (25 μg/ml), transferrin (50 μg/ml), progesterone (20 nM), putrescine (100 μM), selenium chloride (30 nM) plus FGF2 (10 ng/ml), and laminin (1 μg/ml). After 5 days, cells were harvested by scraping in calcium- and magnesium-free Hanks' buffered salt solution (CMF-HBSS), triturated to a single-cell suspension, replated at a 1:5 ratio, and grown to subconfluency in the presence of FGF2 (10 ng/ml) and EGF (20 ng/ml). Cells were then passaged at a 1:5 ratio and again grown to subconfluency in the presence of FGF2 (10 ng/ml) and PDGF-AA (10 ng/ml). Human recombinant FGF2, EGF, and PDGF-AA (R&D Systems, Minneapolis, MN) were added daily and the medium was replaced every 2 days. In vitro differentiation was induced by growth factor withdrawal.
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13. Cells growing in medium containing FGF2 and PDGF were harvested and triturated to a single-cell suspension. Spinal cord transplants were performed as described (*23*). Following laminectomy at the thoracolumbar transition, 100,000 cells in a volume of 1 μl CMF-HBSS were injected into the dorsal column of 7-day-old rnd rats at one or several levels. The recipient animals received daily i.p. injections of cyclosporin (10 mg/kg body weight). All procedures were done in accordance with institutional guidelines.
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15. Transplant recipients were perfusion-fixed with 4% paraformaldehyde at 3 weeks of age. Brains and spinal cords were processed for vibratome sectioning. Selected spinal cord transplant recipients were processed for semi- and ultrathin sectioning by routine procedures. Primary antibodies used for immunohistochemistry were anti-PLP (1:500), anti-MBP (Boehringer; 1:200), anti-CNP (Sigma; 1:200), and anti-GFAP (ICN; 1:100). Antigens were visualized using appropriate peroxidase-conjugated secondary antibodies. Identification of donor cells by DNA in situ hybridization with a probe to mouse satellite DNA was done as described (*16*).
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Two-Metal-Ion Catalysis in Adenylyl Cyclase

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Adenylyl cyclase (AC) converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate, a ubiquitous second messenger that regulates many cellular functions. Recent structural studies have revealed much about the structure and function of mammalian AC but have not fully defined its active site or catalytic mechanism. Four crystal structures were determined of the catalytic domains of AC in complex with two different ATP analogs and various divalent metal ions. These structures provide a model for the enzyme-substrate complex and conclusively demonstrate that two metal ions bind in the active site. The similarity of the active site of AC to those of DNA polymerases suggests that the enzymes catalyze phosphoryl transfer by the same two-metal-ion mechanism and likely have evolved from a common ancestor.

Mammalian ACs are integral membrane proteins that catalyze the synthesis of the second messenger adenosine 3′,5′-monophosphate (cAMP) from ATP. Many signaling pathways converge on and regulate the enzyme (*1*), including those of heterotrimeric GTP-binding proteins (G proteins), which couple the activation of heptahelical receptors on the cell surface to subsequent changes in AC activity. G proteins bind directly to the catalytic core of AC, which consists of two homologous cytoplasmic domains, C_{1a} and C_{2a}. These domains can be expressed as independent polypeptides in *Escherichia coli* and, when mixed, form a heterodimer that exhibits catalytic activity as well as sensitivity to $G_s\alpha$ and $G_i\alpha$, the stimulatory and inhibitory G protein α subunits (*2–4*).

We recently determined the crystal structure of a complex between $G_s\alpha$ and the C_{1a}:C_{2a} heterodimer (Fig. 1A) and proposed a

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