Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes

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Stable clones of neural stem cells (NSCs) have been isolated from the human fetal telencephalon. These self-renewing clones give rise to all fundamental neural lineages in vitro. Following transplantation into germinal zones of the newborn mouse brain they participate in aspects of normal development, including migration along established migratory pathways to disseminated central nervous system regions, differentiation into multiple developmentally and regionally appropriate cell types, and nondisruptive interspersion with host progenitors and their progeny. These human NSCs can be genetically engineered and are capable of expressing foreign transgenes in vivo. Supporting their gene therapy potential, secretory products from NSCs can correct a prototypical genetic metabolic defect in neurons and glia in vitro. The human NSCs can also replace specific deficient neuronal populations. Cryopreservable human NSCs may be propagated by both epigenetic and genetic means that are comparably safe and effective. By analogy to rodent NSCs, these observations may allow the development of NSC transplantation for a range of disorders.

Keywords: cell therapy, progenitor cell, gene therapy, Tay-Sachs disease, transplanation, differentiation

Neural stem cells (NSCs) are primordial, uncommitted cells postulated to give rise to the array of more specialized cells of the central nervous system (CNS)[1–9]. They are operationally defined by their ability (1) to differentiate into cells of all neural lineages (i.e., neurons—ideally of multiple subtypes, oligodendroglia, astroglia) in multiple regional and developmental contexts; (2) to self-renew (to give rise to new NSCs with similar potential); and (3) to populate developing and/or degenerating CNS regions. The demonstration of a monoclonal derivation of progeny is obligatory to the definition (i.e., a single cell must possess these attributes). With the earliest recognition that rodent neural cells with stem cell properties, propagated in culture, could be reimplanted into mammalian brain where they could reintegrate appropriately and stably express foreign genes[10–13], gene therapists and neurobiologists began to speculate how such a phenomenon might be harnessed for therapeutic advantage as well as for understanding developmental mechanisms. These, and the studies they spawned (reviewed in refs. 14–16), provided hope that the use of NSCs might circumvent some limitations of presently available graft material[16] and gene transfer vehicles[17] and make feasible a variety of therapeutic strategies.

Neural cells with stem cell properties have been isolated from the embryonic, neonatal, and adult rodent CNS and propagated in vitro by a variety of equally effective and safe means—both epigenetic (with mitogens such as epidermal growth factor [EGF][5] or basic fibroblast growth factor [bFGF][13,18,19] or with membrane substrates[7]) and genetic (with propagating genes[15] such as *v-myc*[10,20] or large T-antigen [*T-Ag*][9]). Maintaining NSCs in a proliferative state in culture does not subvert their ability to respond to normal developmental cues in vivo following transplantation (such as the ability to withdraw from the cell cycle, interact with host cells, and differentiate[1,8]). These extremely plastic cells migrate and differentiate in a temporally and regionally appropriate manner particularly following implantation into germinal zones. Intermingling nondisruptively with endogenous progenitors, responding similarly to local cues for their phenotypic determination, and appropriately differentiating into diverse neuronal and glial types, they participate in normal development along the rodent neuraxis. In addition, they can express foreign genes in vivo[8–16], often in widely disseminated CNS regions[21,22], and are capable of neural cell replacement[8].

The presumption has been that the biology that endows such rodent cells with their therapeutic potential is conserved in the human CNS. If true, then progress toward human applications may be accelerated. We demonstrate the potential of clones of human NSCs to perform these critical functions in vitro and in vivo in a manner analogous to their rodent counterparts.

Results and discussion

Isolation, propagation, and cloning of human NSCs. The isolation, propagation, characterization, cloning, and transplantation of NSCs from the human CNS mirrored strategies used for the murine NSC clone C17.2 (propagated following transduction of a constitutively downregulated *v-myc*[10,21]) and for growth factor-expanded murine NSC clones[1,5]. NSCs—even genetically propagated clones[23]—require molecules like bFGF and/or EGF in serum-free medium to divide[18,24]. Therefore, this dual responsiveness was

chosen for both screening and enriching a starting population of stable, dissociated, cultured primary human neural tissue for cells. Cells dissociated from human fetal telencephalon—particularly the ventricular zone, which has been postulated to harbor (in lower mammals) a rich NSC population[7]—were initially grown as a polyclonal population first in serum-supplemented and then in serum-free medium containing bFGF and/or EGF. Cells were transferred between media containing one or the other of the mitogens to select for dual responsiveness. Some populations were then maintained in bFGF alone for subsequent manipulation and cloning; others were used for retrovirally mediated transduction of *v-myc* and subsequent cloning.

To provide an unambiguous molecular tag for assessing the clonal relationships of the cells, as well as to facilitate identification of some cells following transplantation and to assess their capacity to express exogenous genes in vivo, some bFGF-propagated subpopulations were infected with an amphotropic replication-incompetent retroviral vector encoding *lacZ* (and *neo* for selection). Single resistant colonies were initially isolated by limiting dilution. Monoclonality of the cells in a given colony was then confirmed by demonstrating the presence of only one copy of the *lacZ/neo*-encoding retrovirus, with a unique chromosomal insertion site. In clone H1, for example, all *lacZ/neo*-positive cells, had a single, common retroviral integration site indicating that they were derived from a single infected "parent" cell (Fig. 1A).

In rodents, genes (such as *v-myc* and *T-Ag*) that interact with cell cycle regulatory proteins have been used to propagate NSCs[8,9], neural progenitors[25], and neuroblasts[12], resulting in engraftable rodent NSC clones that can be manipulated and have therapeutic potential[14]. Therefore, some of the bFGF-maintained human cell populations, enriched for NSCs, were infected with an amphotropic, replication-incompetent retroviral vector encoding *v-myc* and *neo*[20] yielding multiple colonies. All of the putative clones had only one unique retroviral insertion site, demonstrating their monoclonality (Fig. 1B). Five clones (H6, H9, D10, C2, and E11) were generated and maintained in serum-free medium containing bFGF.

Multipotency and self-renewal in vitro. In uncoated dishes and in serum-free medium supplemented with bFGF, all clones grew in culture as clusters that could be passaged weekly for at least 1 year (Fig. 2A). The cells within these clusters expressed vimentin, a neural progenitor marker[18]. By dissociating these clusters and plating them in serum-containing medium, these clones differentiated spontaneously into neurons and oligodendrocytes (Figs. 2B and C). After 5 days under these differentiating conditions, 90% of the cells in all clones became immunoreactive for the neuronal marker neurofilament (NF; Fig. 2B); 10% expressed CNPase, a marker for oligodendroglia (Fig. 2C). Mature astroglia containing glial fibrillary acidic protein (GFAP) were not initially observed, even after 1 month under these culture conditions. However, GFAP production could be induced by coculture with primary dissociated embryonic murine CNS tissue (Fig. 2D). In addition to cells expressing the variety of differentiated lineage-specific markers (establishing "multipotency"), each clone gave rise to new immature vimentin-positive cells (Fig. 2E), which could, upon subsequent passage, give rise to new cells expressing multiple differentiated neural markers as well as to new vimentin-positive passageable cells (i.e., "self-renewability"). All the clones, whether genetically modified or epigenetically maintained, were similar in vitro.

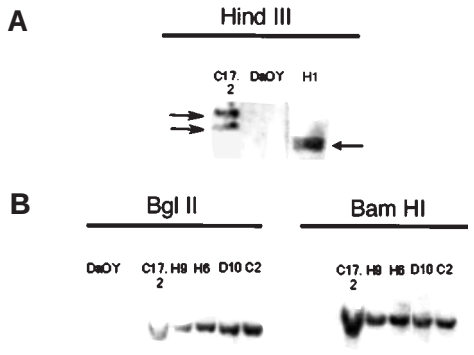

Figure 1. Southern blot analysis of retroviral insertion into human NSC clones. (A) Genomic DNA from clone H1 (propagated in bFGF and transduced with a retrovirus encoding *lacZ* and *neo*) digested with Hind III (cuts once within the provirus) and incubated with a radiolabeled *neo* probe. The murine NSC clone C17.2 contains two integrated proviruses encoding *neo*[10,20]. DaOY is an uninfected human meduloblastoma cell line. (B) Genomic DNA from clones H9, H6, D10, and C2 (propagated in bFGF and/or EGF and infected with a retrovirus encoding *v-myc*) were digested with BglII or BamHI (cuts once within the provirus) and probed for *v-myc*. C17.2 contains one *v-myc*-encoding provirus.

Ability to cross-correct a genetic defect. To assess their potential as vehicles for molecular therapies, we compared the ability of human NSCs to complement a prototypical genetic defect to murine NSCs[26]. The neurogenetic defect chosen was in the α-subunit of β-hexosaminidase, a mutation that leads to hexosaminidase-A deficiency and a failure to metabolize GM_2 ganglioside to GM_3 (Tay-Sachs disease [TSD]). Pathologic GM_2 accumulation in the brain leads to progressive neurodegeneration. The ability of human NSCs to cross-correct was compared with that of two established murine NSC clones: C17.2 and a subclone of C17.2 (C17.2H) engineered via retroviral transduction of the human α–subunit gene to overexpress hexosaminidase[22]. These murine NSC clones secrete functional hexosaminidase-A[22]. A transgenic mouse with an α-subunit deletion[27] permitted examination of the ability of human NSCs to secrete a gene product capable of rescuing TSD neural cells. NSCs (murine and human) were cocultured with dissociated TSD mouse brain cells from which they were separated by a porous membrane that allowed passage of hexosaminidase but not cells. After 10 days, the mutant neural cells were examined: (1) for the presence of hexosaminidase activity (Fig. 3A–C, and M); (2) with antibodies to the α-subunit and to CNS cell type markers to determine which TSD neural cells internalized corrective gene product (Fig. 3D–L); and (3) for reduction in GM_2 storage (Fig. 3N). While there was minimal intrinsic hexosaminidase activity in TSD cells cultured alone (Fig. 3A), hexosaminidase activity increased to normal intensity when the cells were cocultured with murine or human NSCs (Fig. 3B and C). The extent of human NSC-mediated cross-correction matched the success of murine NSCs, yielding percentages of hexosaminidase-positive TSD cells significantly greater than in untreated controls ($p<0.01$) (Fig. 3M). All neural cell types from the TSD mouse brain were corrected (Figs. 3D–L). The percentage of TSD CNS cells without abnormal GM_2 accumulation was significantly lower in those exposed to secretory products from human NSCs than in untreated TSD cultures ($p<0.01$), approaching those from wild-type mouse brain (Fig. 3N).

Multipotency and plasticity in vivo. We next determined whether human NSC clones (whether epigenetically or genetically propagated) could respond appropriately to normal developmental cues in vivo, which include migrating appropriately; integrating into host parenchyma; and differentiating into neural cell types appropriate to a given region's stage of development, even if that stage is not the one in which the NSCs were obtained. Although there are many approaches for testing these qualities[28–30], we used paradigms similar to those we have used with murine NSCs to assess their developmental ability[8]. When murine NSC clones are implanted into the cerebral ventricles of newborn mice, the cells engraft in the subventricular germinal zone (SVZ)[31] and follow the

established pathways used by endogenous progenitors, either migrating along the rostral migratory stream (RMS) to the olfactory bulb (OB), becoming neurons[32], or migrating into subcortical and cortical regions (where gliogenesis predominates and neurogenesis has ceased) becoming oligodendroglia and astroglia[33]. When transplanted into the germinal zone of the neonatal mouse cerebellum (the external germinal layer [EGL]), these same NSCs migrate inward and differentiate into granule neurons in the emerging internal granule cell layer (IGL)[10]. Following intraventricular implantation, human NSC clones emulated the developmentally appropriate behavior of their murine counterparts (Fig. 4 and 5). The engraftment, migration, and differentiation of epigenetically perpetuated clones were identical to that of *v-myc* perpetuated clones. Three of the five *v-myc* clones engrafted well (Table 1).

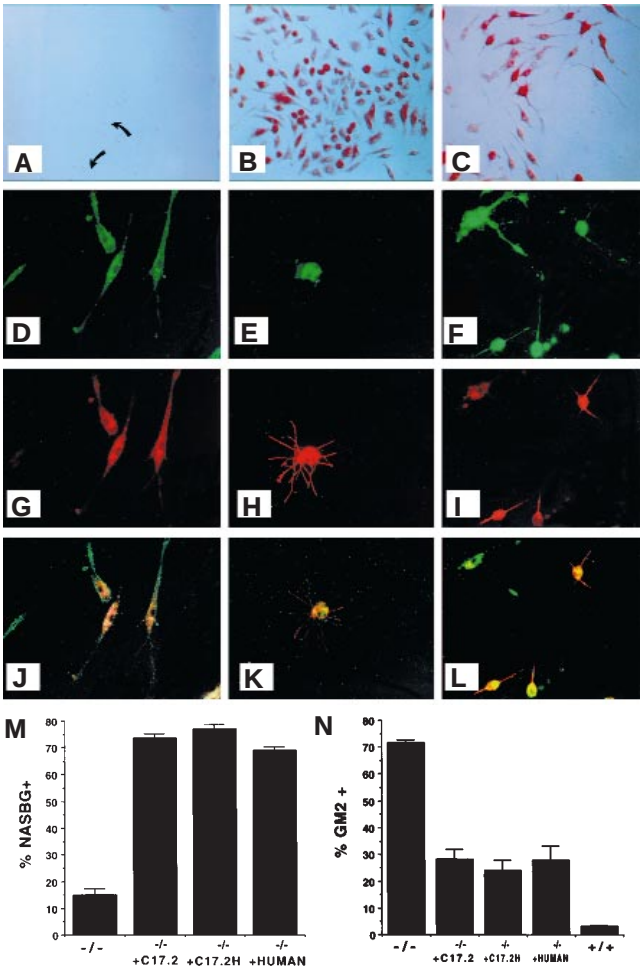

Figure 3. Dissociated brain cells from mice with mutated α–subunit of β-hexosaminidase (Tay-Sachs disease) cocultured with human NSCs. (A–C) Hexosaminidase activity determined by NASBG histochemistry. (A) TSD neural cells (arrows) not exposed to NSCs. TSD cells exposed to secretory products from (B) murine NSC clone C17.2H or from (C) human NSCs. (D–L) TSD cells cocultured with human NSCs immunostained with a (D–F) fluorescein-labeled antibody to the human α-subunit of β-hexosaminidase and (G–I) with antibodies to neural cell type-specific antigens. (G) Neuronal-specific NeuN marker; (H) glial specific GFAP marker; and (I) precursor maker, nestin. (J–L) Dual filter microscopy of the α-subunit and cell-type markers. (M) Percentage of β-hexosaminidase positive TSD cells; -/-: TSD α-subunit–null cells; TSD cells exposed to secretory products from C17.2+ murine NSCs; C17.2H+: murine NSC engineered to overexpress murine hexosaminidase; +human: human NSCs. (N) GM_2 accumulation in TSD cells; labels as in (M); +/+: wild-type mouse brain.

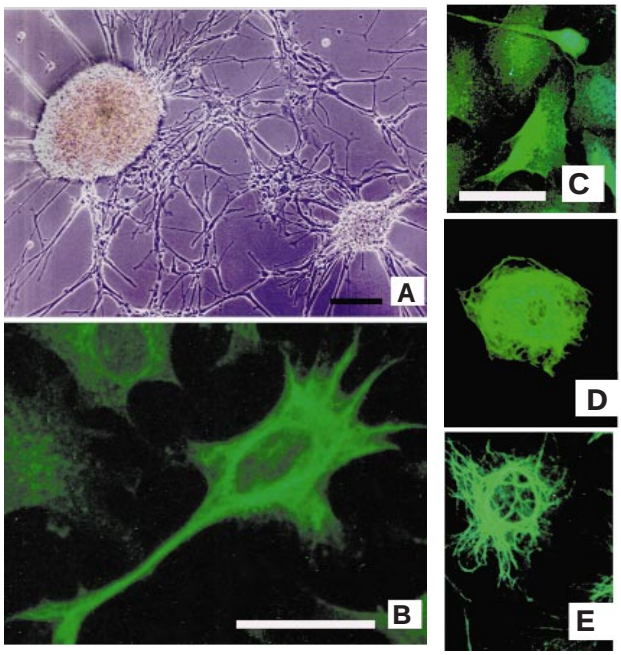

Figure 2. Characterization of human NSCs in vitro. (A) NSCs grown in serum-free medium. Immunostaining for (B) the neuronal marker neurofilament or (C) the oligodendroglia marker CNPase in serum-containing medium. (D) Immunostaining for the astrocyte marker human GFAP upon coculture with primary murine CNS cultures. (E) Immunostaining for the immature neural marker vimentin at transfer to serum-containing medium.

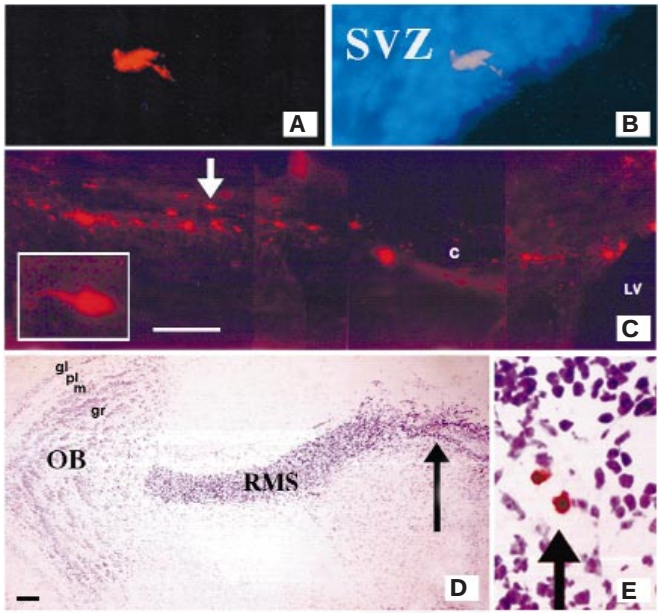

Figure 4. Migration of human NSCs following engraftment into the SVZ of newborn mice. (A,B) Human NSCs 24 h after transplantation. (A) Donor-derived cell (red) interspersed with (B) densely packed endogenous SVZ cells, visualized by DAPI (blue) in the merged image. (C) Donor-derived cells (red) within the subcortical white matter (arrow) and corpus callosum (c) and their site of implantation in the lateral venticles (LV). Arrow indicates the cell shown at higher magnification within the inset. (D) Donor-derived cell migration from the SVZ into the rostral migratory stream (RMS) leading to the olfactory bulb (OB), in a cresyl-violet counterstained parasagittal section; gl: glomerular layer; pl: plexiform layer; m: mitral layer; gr: granular layer. Scale bars: 100 μm. (E) Higher magnification of area indicated by the arrow in (D). Brown staining indicates BrDU-immunoperoxidase-positive donor-derived cells.

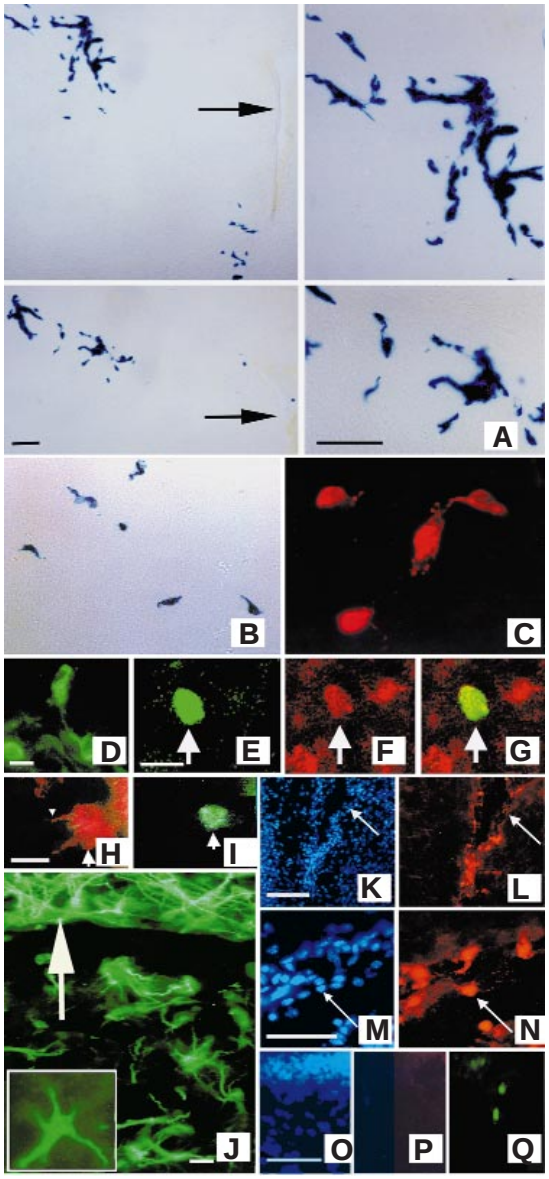

Figure 5. Characterization of human NSC clones in vivo following engraftment into SVZ of neonatal mice. (A–C) *LacZ*-expressing donor-derived cells from human NSC clone H1 detected with (A,B) Xgal and with (C) anti-β-galactosidase within (A) the periventricular and subcortical white matter regions and (B,C) OB granule layer. The arrows in (A) indicate the lateral ventricles. (D–G) BrdU-labeled NSCs (clone H6) implanted into the SVZ at birth identified in the OB with a (D) human-specific NF antibody and by (E–G) BrdU ICC via confocal microscopy. (E) BrdU-positive cell visualized by fluorescein; (F) anti-NeuN+ antibody visualized by Texas Red; (G) same cell visualized by dual filter. Donor-derived clone H6 in the adult subcortical white matter double-labeled with (H) an oligodendrocyte-specific antibody to CNPase and (I) BrdU. The arrowhead in (H) indicates a cytoplasmic process extending from the soma. (J) Donor-derived astrocytes (clone H6) in the adult subcortical white matter (indicated by the arrow) and striatum following neonatal intraventricular implantation, immunostained with a human astrocyte-specific anti-GFAP antibody. Inset is higher magnification. (K–Q) Expression of *v-myc* by human NSC clone H6 (K–N) 24 hours and (O–Q) 3 weeks following engraftment in the SVZ. (K,M,O) DAPI nuclear stains of the adjacent panels (L,N,P), immunostained for *v-myc* and (Q) immunostained for BrdU-positive donor-derived cells. (Q) is same as (P). Scale bars: (A and K): 100 μm; (D and E): 10 μm; (O): 50 μm.

Human NSCs integrated into the SVZ within 48 h following implantation (Figs. 4A and B, 5K–N). As with endogenous SVZ progenitors, engrafted human NSCs migrated out along the subcortical white matter by 2 weeks following engraftment (Fig. 4C), and, by 3–5 weeks had appropriately differentiated into oligodendrocytes and astrocytes (Fig. 5A and H–J). The ready detection of donor-derived astrocytes in vivo (Fig. 5J) contrasts with the initial absence of mature astrocytes when human NSC clones were maintained in vitro in isolation from the in vivo environment (Fig. 2D). Signals emanating from other components of the murine CNS appear necessary for promoting astrocyte differentiation and/or maturation from multipotent cells.

Endogenous SVZ progenitors also migrate anteriorly along the RMS and differentiate into OB interneurons[35]. By 1 week following transplantation, a subpopulation of donor-derived human cells from the SVZ migrated along the RMS (Fig. 4D and E). In some cases, these cells migrated together in small groups (Fig. 4E), a

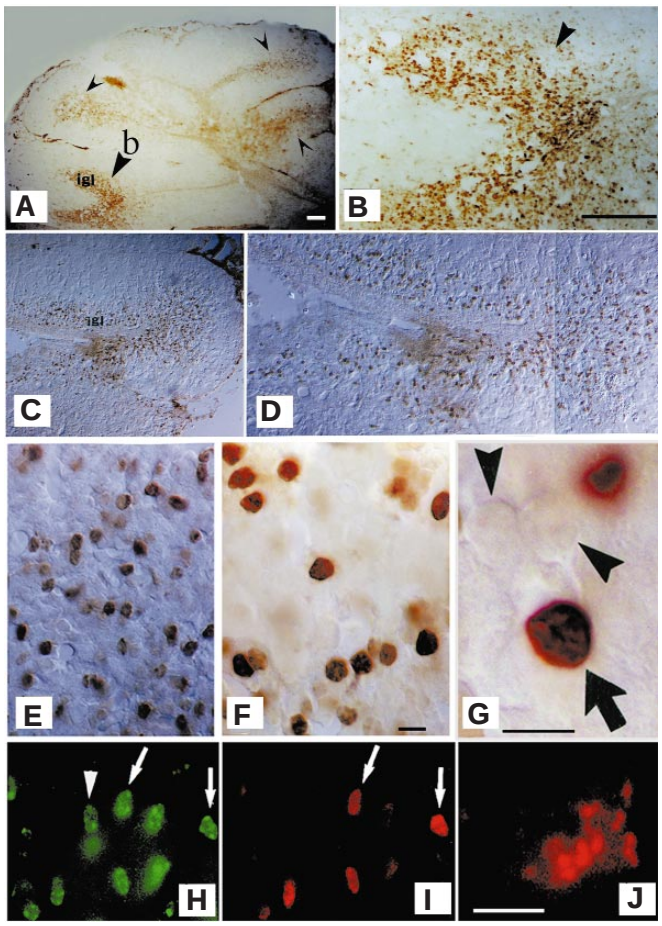

Figure 6. Transplantation of human NSCs into granule neuron-deficient cerebellum. (A–G) Donor-derived cells (clone H6) identified in the mature cerebellum by anti-BrDU immunoperoxidase cytochemistry (brown nuclei) following implantation into and migration from the neonatal *mea* EGL. (A) The internal granule cell layer (IGL and arrowheads) within the parasagittal section of the cerebellum. (B) Higher magnification of the posterior lobe indicated by "b" in (A). (C–G) Increasing magnifications of donor-derived cells within the IGL of a *mea* anterior lobe (different animal from [A,B]). (G) Normarski optics: residual host granule neurons indicated by arrowheads, representative BrDU positive donor-derived neuron indicated by the arrow. (H) Colabeling with anti-BrDU (green) and (I) NeuN (red) indicated with arrows. Arrowhead indicates BrDU+/NeuN- cell. (J) Fluorescent in situ hybridization of cells within the IGL using a human-specific probe (red). Scale bars: (A and B): 100 μm; (F, G, and J): 10 μm.

Table 1. Human neural stem cell clones.

Clone	Propagation technique	Engraftable
H1	bFGF	+
H6	v-*myc*	+
H9	v-*myc*	+
E11	v-*myc*	+
D10	v-*myc*	–
C2	v-*myc*	–

behavior typical of endogenous murine SVZ precursors[32]. Three weeks following transplantation, a subpopulation of donor-derived neurons (human-specific NF-positive cells) were present within the parenchyma of the OB, intermingled with host neurons (Fig. 5B–G). Not only were these donor-derived cells human NF-positive (Fig. 5D), but, when sections through the OB were reacted with both an antibody against BrDU (to identify prelabeled donor-derived human cells) and with an antibody to the mature neuronal marker NeuN, a large number of double-labeled BrDU+/NeuN+ donor-derived cells were integrated within the granule layer (Fig. 5E–G), mimicking the NeuN expression pattern of endogenous, host, murine interneurons (Fig. 5F and G).

Identical clones were implanted into a different germinal zone at the opposite end of the neuraxis to determine their plasiticity. Transplants of the same human NSCs into the EGLs of newborn mouse cerebella appropriately yielded different neuronal cell types in this different location, primarily cerebellar granule cells in the IGL (Fig. 6A–I), detailed below.

Therefore, in vivo—as in vitro (Fig. 2)—all engraftable human NSC clones gave rise to cells in all three fundamental neural lineages: neurons (Figs. 5D–G and 6], oligodendrocytes (Fig. 5H and I), and astrocytes (Fig. 5J). Not only did transplanted brains look histologically normal (donor cells migrated and integrated seamlessly into host parenchyma yielding no discernible graft margins), but engrafted animals exhibited no indications of neurologic dysfunction. Thus, structures that received contributions from donor human NSCs appeared to have developed normally.

Although most clones engrafted well, two appeared to engraft poorly (Table 1). Nevertheless, in vitro these clones displayed characteristics seemingly identical to those of the more robustly engrafting clones. Thus, ostensibly equivalent multipotency in vitro does not necessarily translate into equivalent potential in vivo, suggesting that each clone should be individually tested. This observation also suggests that transplantation of mixed polyclonal populations, because of their shifting representations of various clones, may be a problematic strategy.

Foreign transgene expression in vivo. Many CNS gene therapy needs require that donor cells express foreign genes in widely disseminated locations[26] (in addition to being able to do so in anatomically restricted regions[14]). Murine NSC clones have this capacity[21,22]. Human NSCs appear similarly capable. A representative retrovirally transduced, *lacZ*-expressing clone (Fig. 5A–C) continued to produce β–galactosidase after migration to, and stable integration and maturation within, host parenchyma at distant sites in the mature animal.

Spontaneous constitutive downregulation of *v-myc* expression. In the case of genetically manipulated human NSC clones, the propagating gene product *v-myc* is undetectable in donor human cells beyond 24–48 h following engraftment (Fig. 5K–Q) despite the fact that the brains of transplant recipients contain numerous stably engrafted, healthy, well-differentiated, nondisruptive, donor-derived cells (Figs. 4, 5A–J and Q, and 6). Identical findings have been observed with *v-myc*–propagated murine NSC clones[8] in which *v-myc* downregulation occurs constitutively and spontaneously and correlates with the typical quiescence of engrafted cells within 24–48 h posttransplantation. These observations suggest that *v-myc* is regulated by the normal developmental mechanisms that downregulate endogenous cellular *myc* in CNS precursors during mitotic arrest and/or differentiation. The loss of *v-myc* expression from stably engrafted NSCs following transplantation is consistent with the invariant absence of brain tumors derived from implanted *v-myc*–propagated NSCs, even after several years in mice[8,10]. As with mouse NSCs, neoplasms are never seen using human NSCs.

Neural cell replacement in vivo. Neurologic mouse mutants have provided ideal models for testing specific neural cell replacement strategies. The *meander tail* (*mea*) mutant is one such model of neurodegeneration and impaired development. *Mea* is characterized by a cell-autonomous failure of granule neurons to develop and/or survive in the cerebellum, especially in the anterior lobe[34]. Murine NSCs are capable of reconstituting the granule neuron-deficient IGL[34]. To assess whether human cells may be comparably effective in replacing neurons in CNS disorders, human NSC clones were engrafted into EGLs of newborn *mea* cerebella. When analyzed at the completion of cerebellar organogenesis, donor-derived human cells were present throughout the IGL (Fig. 6). They possessed the definitive size, morphology, and location of cerebellar granule neurons (Fig. 6E–G), identical to the few residual endogenous murine host granule neurons with which they were intermixed (Fig. 6G). That these replacement neurons were of human origin was confirmed by fluorescence in situ hybridization (FISH), using a human-specific chromosomal probe (Fig. 6J). The neuronal phenotype was confirmed by demonstrating that most engrafted cells in the *mea* IGL were immunoreactive for NeuN (Fig. 6H and I); as in the OB, endogenous interneurons in the IGL similarly express NeuN. Thus, engrafted NSCs of human origin appear sufficiently plastic to respond appropriately to varying local cues for lineage determination; recall that the donor human cells were not initially derived from a postnatal brain or from a cerebellum. Furthermore, human NSCs may be capable of appropriate neural cell replacement, much as murine NSCs are[34,35]. While many gene therapy vehicles depend on relaying new genetic information through established neural circuits—that may, in fact, have degenerated—NSCs may participate in the reconstitution of these pathways.

We have presented evidence that neural cells with stem cell features may be isolated from human brains and emulate NSCs in lower mammals[14], vouchsafing conservation of neurodevelopmental principles and suggesting that this cell type may be applied to a range of research and clinical problems in humans. NSCs may serve as adjuncts to other cellular[16], viral[17], and nonviral[17] vectors, including other human-derived neural cells[36–39]. Not only might the clones described here serve these functions, but our data suggest that investigators may readily utilize NSCs from other human material via a variety of equally safe and effective epigenetic and genetic means. That the methods used here yielded comparable cells suggests that investigators may choose the technique that best serves their needs. Insights from studies of NSCs perpetuated by one strategy may be legitimately joined to those derived from studies using others, providing a more complete picture of NSC biology and its applications.

Experimental protocol

Maintenance and propagation of human NSCs in culture. A suspension of primary dissociated neural cells (5×10^5 cells/ml), initially prepared and stably cultured from the periventricular region of the telencephalon of a 15-week human fetus[36] was plated on uncoated tissue culture dishes (Corning, Cambridge, MA) in the following growth medium: Dulbecco's Modified Eagles Medium (DMEM) + F12 medium (1:1) supplemented with N2 medium (Gibco, Grand Island, NY) to which was added bFGF (10–20 μg/ml) + heparin (8 μg/ml) and/or EGF (10–20 μg/ml). Medium was changed every 5

days. Cell aggregates were dissociated in trypsin-EDTA (0.05%) when >10 cell diameters in size and replated in growth medium at 5 x 10^5 cells/ml.

Differentiating culture conditions. Dissociated NSCs were plated on poly-L-lysine (PLL)-coated slides (Nunc, Naperville, IL) in DMEM + 10% fetal bovine serum (FBS) and processed weekly for immunocytochemistry (ICC). In most cases, differentiation occurred spontaneously. For astrocytic maturation, clones were cocultured with primary dissociated embryonic CD-1 mouse brain[20].

Retrovirus-mediated gene transfer. Two xenotropic, replication-incompetent retroviral vectors were used to infect human NSCs. A vector encoding *lacZ* was similar to BAG[10,20] except for the PG13 xenotropic envelope. An amphotropic vector encoding *v-myc* was generated using the ecotropic vector described for generating murine NSC clone C17.2 (ref. 20) to infect the GP + envAM12 amphotropic packaging line[37]. No helper virus was produced. Infection of bFGF- and/or EGF-maintained human neural cells with either vector (4×10^5 colony-forming units) was as described[10,20].

Cloning of human NSCs. Cells were dissociated, diluted to 1 cell/15 µl and plated at 15 µl/well of a Terasaki or 96-well dish. Wells with single cells were immediately identified. Single-cell clones were expanded and maintained in bFGF-containing growth medium. Monoclonality was confirmed by identifying a single and identical genomic insertion site by Southern blot analysis for either the *lacZ*- or the *v-myc*–encoding provirus in all progeny as described[20]. The *v-myc* probe was generated by nick translation labeling with ^{32}P dCTP; a probe to the *neo* sequence of the *lacZ*-encoding vector was generated by PCR using ^{32}P dCTP.

Cryopreservation. Trypsinized human cells were resuspended in a freezing solution comprising 10% dimethyl sulfoxide, 50% FBS, and 40% bFGF-containing growth medium and brought slowly to -140°C.

Cross-correction of mutation-induced β-hexosaminidase deficiency. The murine NSC clones C17.2 and C17.2H (ref. 22) were maintained in similar serum-free conditions as the human cells. NSCs were cocultured in a transwell system with primary dissociated neural cultures[10] from the brains of either wild-type or α-subunit null (TSD) neonatal mice[27]. These cultures were prepared under serum-free conditions, plated onto PLL-coated glass coverslips, and maintained in the medium described for NSCs. To assess production of a secretable gene product capable of rescuing the mutant phenotype, NSCs (murine and human) were cultured on one side of a membrane with 0.4 µm pores (sufficient to allow passage of hexosaminidase but not cells). The membrane was immersed in a well at the bottom of which rested the coverslip. After 10 days, coverslips were examined for hexosaminidase activity; for expression of the α-subunit in cells of various CNS lineages; for reduction in GM_2 storage. Hexosaminidase activity was assayed by standard histochemical techniques using the substrate napthol-AS-BI-*N*-acetyl-β-D-glucuronide (NASBG)[22]; cells stain increasingly pink-red in direct proportion to their enzyme activity. NASBG staining of dissociated wild-type mouse brain cells served as a positive control for both intensity of normal staining and percentage of NASBG-positive cells (~100%). Neural cell types were identified by ICC with antibodies to standard markers: for neurons, NeuN (1:100; gift of R. Mullen, Chemicorp, Temecula, CA); for astrocytes, GFAP (1:500; Sigma, St. Louis, MO); for oligodendrocytes, CNPase (1:500; Sternberger Monoclonals, Baltimore, MD); and for immature undifferentiated progenitors, nestin (1:1000; Pharmingen, San Diego, CA). The α-subunit of human β-hexosaminidase was detected with a specific antibody[22]. Cells were assessed for dual immunoreactivity to that antibody and to the cell type-specific antibodies to assess which TSD CNS cell types had internalized enzyme from human NSCs. Intracytoplasmic GM_2 was recognized by a specific antibody[27].

Transplantation. For some models, each lateral ventricle of cryoanesthetized postnatal day 0 (P0) mice was injected as described[21] with 2 µl of NSCs suspended in phosphate buffered saline (PBS) (4×10^4 cells/µl). For other models, 2 µl of the NSC suspension were implanted into the EGL of each cerebellar hemisphere and the vermis as described[10]. All transplant recipients and untransplanted controls received daily cyclosporin 10 mg/kg given intraperitoneally (Sandoz, East Hanover, NJ) beginning on day of transplant. CD1 and *mea*^{i} mouse colonies are maintained in our lab.

Detection and characterization of donor human NSCs in vivo. Brains of transplanted mice were fixed and cryosectioned as described[10] at serial time points: P1, P2, and weekly through 5 weeks of age. Prior to transplantation, some human cells were transduced with *lacZ*. To control for and circumvent the risk of transgene downregulation, cells were also prelabeled either by in vitro exposure to BrDU (20 µM; 48 h prior to transplantation) and/or with the nondiffusible vital fluorescent membrane dye PKH-26 (immediately prior to transplantation as per Sigma protocol). Engrafted cells were then detected, as appropriate, by Xgal histochemistry[10]; by ICC with antibodies against β-galactosidase[35] (1:1000, XXX, Durham, NC), BrDU (1:10; Boehringer, Indianapolis, IN), human-specific NF (1:150; Boehringer), and/or human-specific GFAP (1:200; Sternberger Monoclonals); by FISH using a digoxigenin-labeled probe complementary to regions of the centromere present uniquely and specifically on all human chromosomes (Oncor, Gaithersburg, MD); and/or by PKH-26 fluorescence (through a Texas Red [TR] filter), with nondiffusibility having been verified for NSCs. Cell type identity of donor-derived cells was also established as necessary by dual staining with antibodies to neural cell type-specific markers: anti-NF (1:250; Sternberger) and anti-NeuN (1:20) to identify neurons; anti-CNPase (1:200–1:500) to identify oligodendrocytes; and anti-GFAP (1:150) to identify astrocytes. Immunostaining used standard procedures[35] and a TR-conjugated secondary antibody (1:200; Vector, Burlingame, CA). Immunoreactivity to human-specific antibodies also used standard procedures and a fluorescein-conjugated antimouse IgG secondary antibody (1:200; Vector). To reveal BrDU-intercalated cells, tissue sections were first incubated in 2N HCl (37°C for 30 min), washed twice in 0.1 M sodium borate buffer (pH 8.3), washed thrice in PBS, and permeabilized before exposure to anti-BrDU. Immunoreactivity was revealed with either a fluorescein-conjugated (1:250; Jackson, West Grove, PA) or a biotinylated (1:200; Vector) secondary antibody. *V-myc* expression (unique to donor-derived cells) was assessed with an antibody to the protein (1:1000; UBI, Lake Placid, NY). To visualize cellular nuclei, sections were incubated in the blue fluorescent nuclear label DAPI (10 min at 20°C). FISH for the human-specific centromere probe was performed on cryosections from 4% paraformaldehyde/2% glutaraldehyde-fixed brains that were permeabilized, incubated in 0.2 N HCl, exposed to proteinase K (100 µg/ml in 0.1M Tris, 0.005 M EDTA [pH 8.0]), washed (0.1% glycine), and rinsed (50% formamide/2× SSC). Probe was then added to the sections, which were coverslipped, denatured (100°C for 10 min), hybridized (15 h at 37°C), and washed (per manufacturer's protocol). Probe was detected by an antidigoxigenin TR-conjugated antibody (Boehringer) diluted 1:5 in 0.5% bovine serum albumin + 5% normal human serum in PBS. For some donor cells, multiple detection techniques were performed.

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