Brain Pathology 9: 569-598(1999)

SYMPOSIUM: Neural Stem Cells

Establishment and Properties of Neural Stem Cell Clones: Plasticity *In Vitro* and *In Vivo*

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The study of the basic physiology of the neural precursors generated during brain development is driven by two inextricably linked goals. First, such knowledge is instrumental to our understanding of how the high degree of cellular complexity of the mature central nervous system (CNS) is generated, and how to dissect the steps of proliferation, fate commitment, and differentiation that lead early pluripotent neural progenitors to give rise to mature CNS cells. Second, it is hoped that the isolation, propagation, and manipulation of brain precursors and, particularly, of multipotent neural stem cells (NSCs), will lead to therapeutic applications in neurological disorders. The debate is still open concerning the most appropriate definition of a stem cell and on how it is best identified, characterized, and manipulated. By adopting an operational definition of NSCs, we review some of the basic findings in this area and elaborate on their potential threapeutic applications. Further, we discuss recent evidence from our two groups that describe, based on that rigorous definition, the isolation and propagation of clones of NSCs from the human fetal brain and illustrate how they have begun to show promise for neural cell replacement and molecular support therapy in models of degenerative CNS diseases. The extensive propagation and engraftment potential of human CNS stem cells may, in the not-too-distant-future, be directed towards genuine clinical therapeutic ends, and may open novel and multifaceted strategies for redressing a variety of heretofore untreatable CNS dysfunctions.

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Introduction

Recent studies have shown that actively proliferating neural precursors persist in specific areas of the mature mammalian central nervous system (CNS). These cells may provide regions such as the hippocampus and the olfactory bulb (OB) with their ability to support local neurogenesis throughout life (1, 10, 74, 131). Unfortunately, except for these "peculiar" areas, virtually no neurogenetic capacity and, consequently, no self-repair or regeneration, are observed following injury or degeneration in most regions of the mature brain. Cell-based therapies, therefore, have emerged as prospective approaches to brain pathologies of diverse etiologies, from metabolic and genetic neurodegenerative disorders to demyelinating diseases and post-traumatic lesions (67, 67a, 110). In essence, the idea underlying the development of cell-based therapies for CNS pathologies is deceivingly simple. The appropriate type of cells would be selected and propagated *ex vivo* and then implanted near or into the damaged brain region where they would replace the missing cells and/or deliver therapeutic molecules. This approach clearly puts emphasis on the availability of large quantities of engraftable cells endowed with appropriate functional characteristics. Effective therapeutic action would seem to require that the engrafted cells not only survive following implantation but that they also: *i.* differentiate (or promote the differentiation of endogenous cells) into the missing cell types; *ii.* intermix seamlessly into the host brain cytoarchitecture; *iii.* display appropriate functional integration into the host neuronal circuitry; and *iv.* steadily secrete therapeutic factors, possibly in a regulated fashion, either spontaneously or following *ex vivo* genetic engineering.

It is not surprising, then, that the last decade has seen the flourishing of studies aimed at isolating primary cell types or at establishing cell lines that may satisfy most of these criteria. Thus, the efficacy of non-neural cells (33), xenogeneic neural cells (20, 142), primary neural tissue (6, 80), genetically-propagated neural precursors (25, 26, 67a, 101, 114), and growth factor-expanded neural cells (75, 116, 131), has been evaluated in transplantation paradigms targeting various experimental models of brain pathology (32, 101, 107, 108, 110). While obtaining and manipulating cells of non-neural origin (e.g., skin fibroblasts, myoblasts) presents obvious practical advantages (e.g., ready availability from biopsies of prospective transplant recipients), their use for intracerebral transplantation is likely going to be limited because they lack the inherent capacity to integrate into the host brain in an appropriate fashion. The most promising candidates for restorative transplantation in the mature CNS are, therefore, cells of neural origin (9). Among the wide array of neural cells available, undifferentiated fetal neural precursors seem to represent the most logical choice. In fact, the developing mammalian CNS embodies an extremely wide array of progenitor cells that are heterogeneous in their lineage specification (i.e. ability to differentiate into various neuronal and glial cell types) and proliferation potential, even within the same brain region. Unfortunately, in the absence of specific and unambiguous cell lineage markers, this wealth of neural cell types rather represents an obstacle to the characterization, isolation and, in the final analysis, handling and exploitation of neural progenitors for specific therapeutic purposes.

The mechanisms underlying the genesis of heterogeneous neural progenitors and the problems related to their identification have been extensively reviewed previously (45, 53, 122). Here, we discuss the isolation and characterization of some primitive neural "ancestors" — identified as "neural stem cells" (NSCs) — in rodents and the recent isolation and unequivocal identification of their human counterparts, illustrating how these observations will likely expedite the progression of intracerebral transplantation from the experimental to the pre-clinical/clinical stage. To put these findings into the appropriate context and to underline the need for a rigorous definition of NSCs that allows for their unambiguous identification, this review also briefly recapitulates some basic concepts concerning the genesis of the heterogeneous array of neural precursor lineages during neurogenesis.

Neural Precursor and Lineage Heterogeneity During Neurogenesis: The "Rising Star" of CNS Stem Cells

One of the most distinguishing characteristics of the mammalian CNS is to be found in its wealth of cellular diversity. Thus, one of the central themes in neurobiology is the study of the fundamental processes by which the bewildering array of mature cells of the adult CNS is generated from the smaller and ostensibly less heterogeneous pool of proliferating progenitors of the primordial neuroepithelium (42).

The study of the basic cellular aspects of the neurogenetic process has been approached by both *in vivo* and *in vitro* analysis and has led to the identification of a wide and heterogeneous array of neural precursors generated at specific stages of development in various CNS regions. In general, the identification/classification of these cells hinges on differences in their functional attributes. In one widely accepted view, neural progenitors are classified with respect to their lineage potential, i.e., their ability to generate one or more the three major mature CNS cell types: neurons, astroglia, and oligodendroglia. Based on this classification scheme, at least three categories of neural cells can be provisionally defined: *1.* "unipotent" progenitors that give rise solely and invariably to neurons (usually a single type of neuron) *or* astroglia *or* oligodendroglia; *2.* "oligopotent" progenitors that generate a broader but nevertheless limited number of neural types (e.g., at least 2 types) — for instance, a number of different neuronal phenotypes but no glia, or one type of neuron and one type of glial cell, or both types glia (astroglia and oligodendroglia) but no neurons, etc.; and *3.* "multipotent" or "pluripotent" progenitors that generate progeny in all three CNS lineages. (For a more detailed discussion of this issue see refs. 45, 53, 122).

In spite of possible regional differences, it is clearly emerging that most, if not all the above types of neural progenitors are generated within all of the regions of the developing neuraxis, though at distinct and specific embryonic ages. For example, in the rodent embryonic day 12-16 (E12-E16) cerebral cortex, the majority of clones detected by retroviral infection studies with reporter genes has been described to be "unipotent" (either neuronal, or astroglial, or oligodendroglial) in origin (61, 62, 87; see also 72) (although the existence of "bipotential" (116, 129) and "multipotential" cells has recently been reported (90, 137)). The embryonic retina and spinal cord appear to contain an opposite ratio: these regions seem to be populated mostly by progenitors that maintain multipotential features throughout development up to their final cell cycle *in vivo* (43,

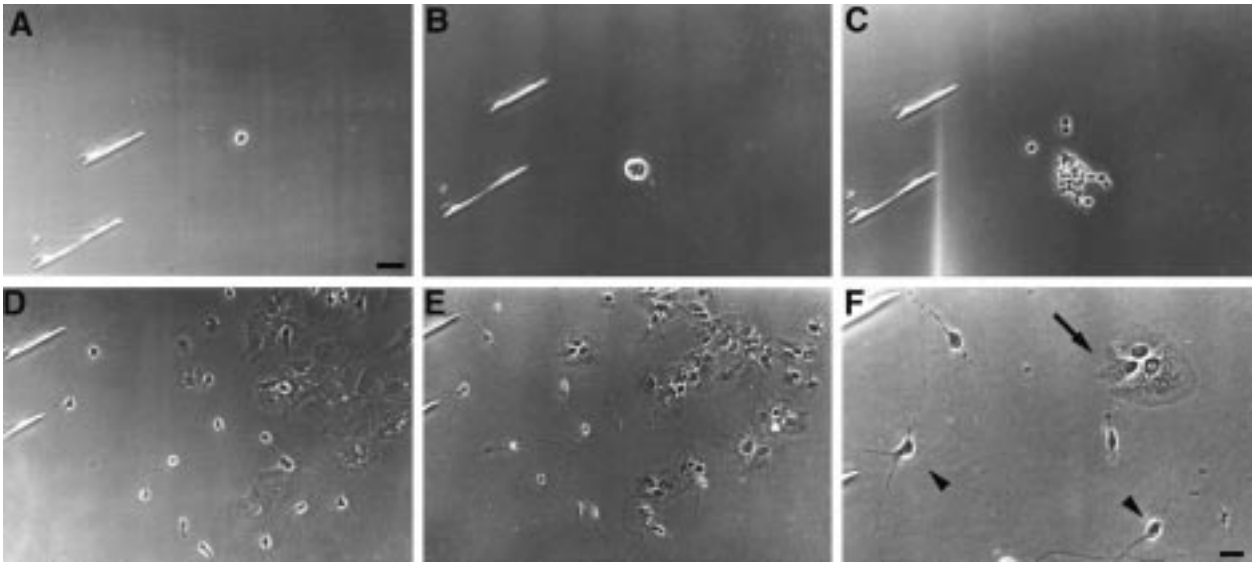


Figure 1. *Lineage restriction of neural stem cell (NSC) progeny in vitro: multipotent EGF-responsive embryonic NSCs generate bipotential (neuronal/astroglial precursors) in vitro.* Stem-like cells isolated from the E14 mouse striatum proliferate, self-renew, and show multilineage capacity when cultured in the presence of high concentrations of EGF (94). A single cell generated by a multipotent EGF-responsive NSC is shown in (**A**), 1 day after plating (DAP) onto a poly-ornithine coated substrate, in the absence of EGF and in the presence of FGF2; the landmark (a scratch on the plate) identifies the field. After 3 DAP, the cell in (**A**) has proliferated (**B**), and by 5 DAP, a small clone of cells has formed (**C**). Addition of fetal calf serum (2% final concentration) triggers differentiation, and, by 6 DAP (**D**), 2 morphologically distinct cell types become apparent which, by 9 DAP (**E**) (high power magnification in (**F**)), acquire the morphology of neurons (arrowheads) and astrocytes (arrow) (see also ref. (126)). Thus, the genesis of neurons and astrocytes from multipotential NSCs may occur through the generation of an intermediate bipotential progenitor cell type. Scale bars: (**A**)-(**E**), 20 μm; (F), 15μm.

50, 124, 125). Yet, unipotent gliablasts that generate solely astroglia or solely oligodendroglia have recently been described in these areas, as well (81).

Although the examples described above are far from exhaustive, they help to underline how the relative representation of distinct precursor subsets may be subject to temporal and spatial variation, and highlight how ill-defined remain the complex genealogical relationships between the different types of neural precursors generated during development. Recent studies have attempted to begin unraveling this complex scenario. Davis and Temple (19) showed, by plating single cells from the dissociated embryonic rodent cerebral cortex in individual tissue culture wells, the presence in this structure of progenitors (albeit only 7% of the clones) that were not only multipotent (i.e., capable of giving rise to neurons, oligodendrocytes, and astrocytes) but that could also reproduce themselves (therefore displaying "self-renewal" capacity). These characteristics are among the defining attributes of "stem cells", i.e. those most primitive of progenitors that are thought to give rise to all of the differentiated cells in most tissues ((55); see also next paragraph). Stem-like cells were also observed in mass cortical cultures (137). Further, it was found that multipotential self-renewing progenitors from the basal embryonic forebrain could generate bipotential (neuronal/ astroglial) (Figure 1) and unipotential (neuronal) (Figure 2) precursors which, in turn, possessed limited proliferative capacity (126). Similar findings were reported in the developing spinal cord (70). In light of these observations, some tentative conclusion might be drawn. While this hypothesis may not be automatically extrapolated to all regions of the developing CNS (45, 53), it is likely that, in many embryonic brain areas, early multipotent neural progenitors undergo progressive restriction of their lineage capacity as development proceeds, until a single, terminal fate is specified. Importantly, as seen in non-neural systems like the blood (89, 132), this developmentally driven lineage restriction may also be associated with a concomitant reduction in the proliferation capacity of the progenitors. Thus, a model of cellular hierarchies seems to emerge in which more specialized (i.e. more differentiated) and less mitotically active cells are progressively generated from more undifferentiated "ancestors" endowed with a higher proliferative potential, until fate

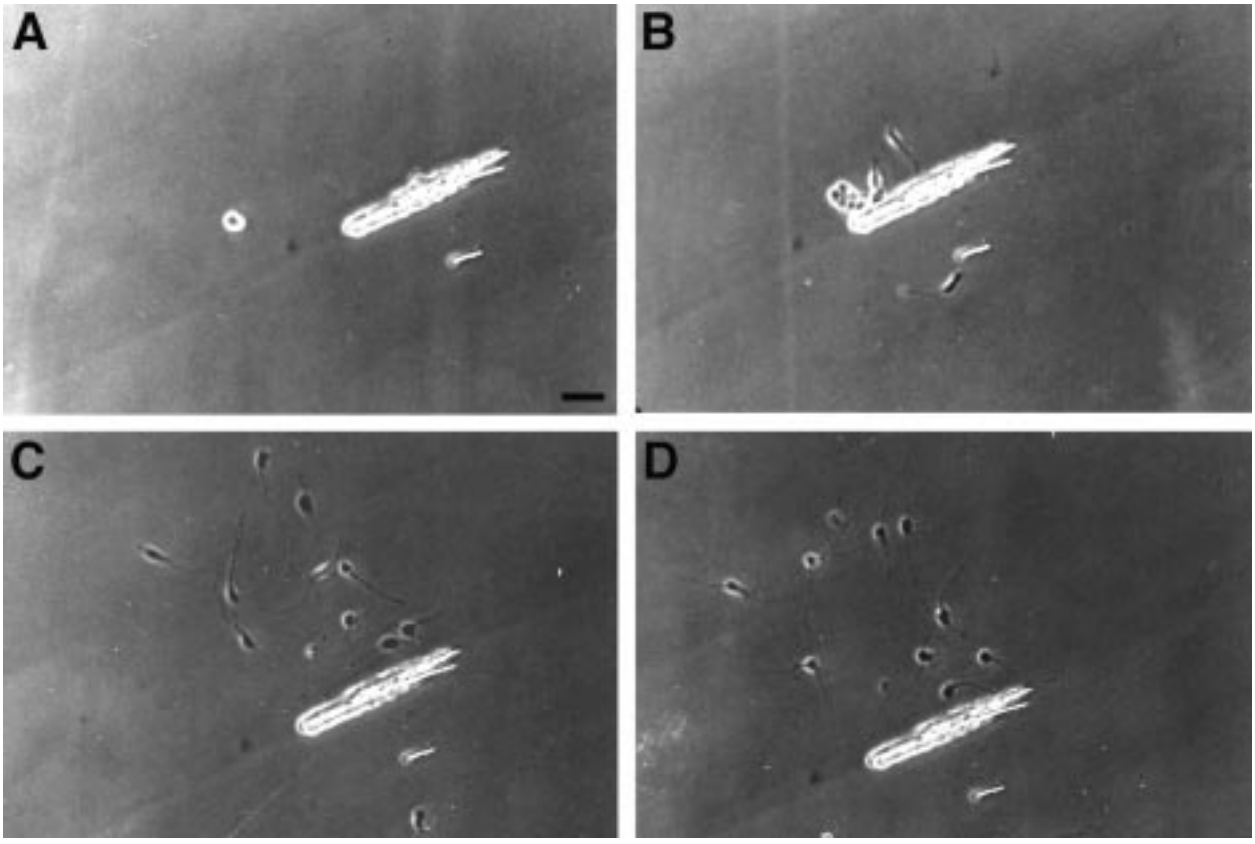


Figure 2. *Lineage restriction of NSC progeny in vitro: multipotential EGF-responsive embryonic NSCs generate unipotential neuronal precursors in vitro.* Unipotential neuronal precursors can also be found among the progeny of multipotential EGF-responsive embryonic NSCs. A single EGF-generated cell is shown 1 DAP in the presence of the sole FGF2. After 5 days, a small clone of 11 cells has formed that, following the addition of FBS (2% final concentration) stop dividing and begin extending processes ((**C**); 7 DAP) and acquire neuronal morphology ((**D**); 9 DAP). These cells express the neuronal marker MAP2 (126).

specification occurs, cell division halts, and terminal differentiation eventually ensues. At the apex of this hierarchy likely sits a distinct set of multipotent elements that are endowed with extensive proliferative, self-renewal, and lineage potential that can span the life of the organism, including into adulthood (55). These cells are defined as "stem cells" and, in the nervous system, as "neural stem cells" (NSCs).

An understanding of the basic physiology of NSCs is essential for their identification, isolation, characterization, and propagation — knowledge that, in turn, is indispensable for exploiting the medical potential rooted in the inherent functional attributes of this peculiar "mother of all neural cell types".

Neural Stem Cells: Definition, Properties and Function

The principal function of a stem cell is to generate all of the differentiated cell types of the tissue in which it resides. During embryonic development, this ability permits the rapid generation of the end-differentiated cells that will form the mature tissue; in adulthood, this capacity should (and, in the hematopoietic system, does) promote replacement of differentiated cells lost to physiological turnover or injury. To fulfill these roles, stem cells are inherently endowed with functional characteristics that also provide a basis for their identification, particularly in systems like the CNS, where specific, unambiguous, reliable stem cell markers have yet to be identified.

Hence, "stem cells" are generally defined operationally as cells that are: *1.* undifferentiated, i.e. lack specific morphology and do not express antigens of mature cells; *2.* proliferating and capable of self-renewal; *3.* multipotential, i.e. can give rise to a wide array of mature, functional progeny *in vitro* and *in vivo*; and *4.* capable of generating progeny that can integrate into and repair the tissue of origin. For a given cell, it is not

always possible to assess all of these parameters simultaneously. As a consequence, different studies have put stronger weight on different functional attributes as a means for confirming stem cell identity, particularly in the CNS.

Likely, the most important characteristic of a stem cell is its self-renewal capacity, i.e., ability to maintain the stem cell population at a steady state or to expand it under certain circumstances. At the single cell level, this can be achieved either by "asymmetric" division, generating one stem cell and one differentiated cell, or by "symmetric" division, where both progeny are identical to the mother stem cell (55). In the former case, the overall stem cell number is maintained, whereas in the latter, it increases. In a stem cell population as a whole, both mechanisms may occur, together with symmetric divisions in which two *non*-stem cell daughters are generated. The balance between the different modes of division within the population determines its maintenance or expansion at each generation and can be genetically or epigenetically regulated. As such, the ability to self-renew is an essential feature of *bona fide* stem cells (75).

Multipotentiality — i.e., the capacity of a single self-renewing progenitor to generate the full array of organ-related lineages — is also a *sine qua non* of a stem cell. For a CNS stem cell, this ability implies that its progeny should include functional neurons, astrocytes, and oligodendrocytes of demonstrable monoclonal derivation. Ideally, this property is illustrated by the capacity of a single cell to replace all lost elements in the tissue of origin. For example, in the hematopoietic system, the stem cell identity of a cell is confirmed by its ability to reconstitute completely a lethally irradiated adult bone marrow. The apparent lack of ready and spontaneous regeneration in the adult CNS has, so far, allowed "a capacity for brain repair" to be downplayed as an essential defining characteristic of a *neural* stem cell. However, of late, studies on putative neural stem cell transplantation in various experimental contexts (67a, 114) has begun to emphasize the importance of this attribute as an identifying stem cell feature even in the CNS.

In summary, a positively identified *neural* stem cell would be a single self-renewing precursor capable of generating progeny that can contribute a sufficient number of new differentiated multiple cell types to the mature CNS. Taken together, these characteristics would seem to endow NSCs with clinical potential because their ability to expand in number should provide a theoretically inexhaustible source of various mature neural cells that, following implantation in the CNS, could replace dead cells of multiple lineages and restore function in the damaged brain or spinal cord.

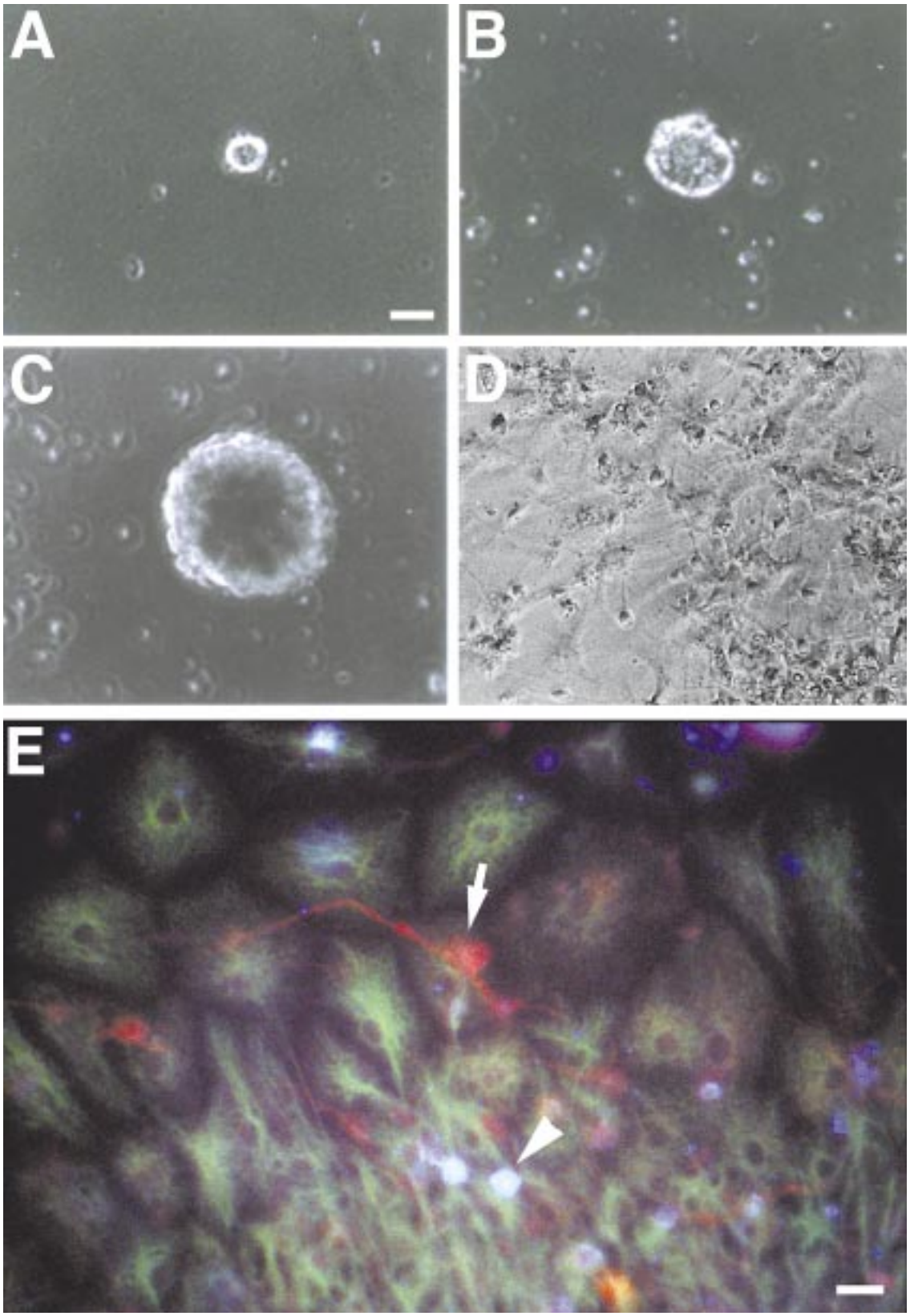
Isolation, Characterization and Propagation of Stem-Like Cells from the Rodent CNS by Epigenetic and Genetic Methods

Due to the long prevailing view that the mammalian CNS is incapable of self-repair/regeneration as well as to technical difficulties in culturing CNS progenitors, the study of stem cells in the mammalian CNS is still in its infancy. As summarized above, the demonstration of stem cell attributes requires the assessment of a series of functional properties in a given cell (55, 73). Although this is greatly facilitated by the observation of isolated cells *in vitro*, NSCs removed from the brain do not normally remain in an undifferentiated, proliferative state in culture; they cease dividing and differentiate after a few, if any, cell cycles (19, 36). To circumvent these limitations and to maintain them in a "stem-like" state, two main approaches have been used: *i.* epigenetic stimulation by chronic exposure of progenitors to mitogenic cytokines or by culturing them on astroglial membrane homogenates, in cellular aggregates, or in the presence of unknown factors contained in chicken extracts (see (53) for review); *ii.* genetic manipulation by transduction of regulated propagation or immortalizing genes into neural progenitors (reviewed in (135)).

Epigenetic stimulation as a means of isolating & propagating multipotent rodent CNS stem-like cells. Various epigenetic approaches have been employed for the isolation and *in vitro* propagation of cells with stem-like properties from the mammalian brain. These methods have included the use of identifiable mitogens like epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2), as well as less defined culture additives like conditioned media and chicken extracts (10, 34, 47a, 74).

A subpopulation of embryonic cortical precursor clones cultured with medium conditioned by astrocytes and meningeal cells was shown unequivocally to be both self-renewing and multipotent (19, 88). Similarly, chicken extract was used to clone multipotent cells from the spinal cord (70).

Cells isolated from both the embryonic and adult basal mouse forebrain (92, 93) and cultured at low plating density in serum-free medium in the presence of high concentrations of EGF initially expressed only nestin, a marker for undifferentiated neuroepithelial cells (51). After prolonged growth and division in culture and with the attainment of higher densities, antigens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indicative of mature neuronal and glial differentiation were observed. Serial subcloning and population analysis eventually confirmed the self-renewal and multilineage potential of these EGF-responsive cells, thus identifying them as genuine NSCs (94).

Cells from the embryonic and adult mammalian forebrain could similarly proliferate in FGF2 and generate differentiated CNS progeny (15, 37, 82, 95). By clonal analysis, some of these cells were shown to bear identifying stem cell features, such as the ability to self-renew, to expand their own population *in vitro*, and to differentiate spontaneously into both glial cell types and electrophysiologically active neurons (Figure 3C) (37). FGF2 could similarly drive the generation of very large clones from the embryonic telencephalon/mesencephalon (46) as well as support neuron- and glia-generating adult hippocampal cells in culture for over 1 year (31).

Together these data suggest that NSCs might exist that somehow differ in their growth factor requirements. For example, while some NSCs proliferate in response to one or the other of the above-mentioned mitogens, NSCs from lower regions of the neuraxis, such as the adult spinal cord, appear in some studies to require simultaneous stimulation by both EGF and FGF2 in order to undergo self-renewal *in vitro* (130).

The maintenance and propagation of NSCs in culture for prolonged periods, as described above, obviously also affords an opportunity to observe and study various aspects of their biology, most particularly their inherent plasticity — a theme to which we will return in discussing other NSC clones *in vitro* and *in vivo* in the sections below. Embryonic EGF-responsive NSCs were noted to generate oligodendrocytes, neurons, and astrocytes in an approximate ratio of 1:5:25, respectively, when allowed to differentiate spontaneously following removal of growth factors in serum-free medium (37, 44, 47a). This differentiation program could be biased, however, by exposure to other factors; for example virtually pure astroglial progeny could be generated by exposing differentiating cells to "bone morphogenic protein 2" (BMP-2) (38). Embryonic and adult FGF2-responsive NSCs could be directed toward the generation of greater numbers of astroglia, neurons, or oligodendroglia depending on whether differentiation occurred in the presence of *i.* ciliary neurotrophic factor (CNTF); *ii*. platelet-derived growth factor (PDGF) or low concentrations of FGF2; or *iii*. thyroid hormone (triiodothyronine (T3)), respectively (44, 88). Exposure to a combination of activin and FGF2 appeared to promote acquisition of a catecholaminergic phenotype by neuronal progeny of EGF-responsive NSCs that might not otherwise display it under standard differentiation conditions (18).

The plasticity of growth factor-responsive CNS stem-like cells has further been suggested by *in vivo* studies. Cells of the SVZ (a repository of presumptive NSCs) have the capacity to contribute new neurons to the to the OB throughout life (i.e., they can replace mature neural cells lost to physiological turnover) (21, 52, 53, 56, 57) and can expand to repopulate regions of an adult rodent SVZ subjected to cytotoxic insult (76, 77). While investigators still debate whether these endogenous cells in the SVZ have a restricted commitment to be OB neurons or possess a broader repertoire (36a, 47, 52, 62, 86), transplantation studies in which well-defined exogenous multipotent NSCs implanted into the SVZ do co-migrate with host SVZ cells to the OB (28) taken together with other studies affirming the ability to obtain such multipotent cells from the SVZ (as described above), do suggest that at least a subpopulation of endogenous SVZ cells have stem-like qualities. Furthermore, it has been demonstrated that the same growth factors that can induce proliferation of cells with stem-like properties *in vitro* (i.e., EGF and FGF2), can also trigger a significant expansion of the SVZ's precursor population following their infusion into the lateral ventricles of the adult mouse. Interestingly, the pattern of SVZ precursor proliferation and migration varies depending on which growth factor is employed. Thus, FGF2 increases the number of neuronal precursors reaching the OB, whereas EGF augments the number of precursor cells found in the brain parenchyma surrounding the ventricles (17, 48).

Preliminary studies suggest the ability of epigenetically-expanded NSCs to engraft and to exhibit plasticity following transplantation into the CNS. For instance, after serial propagation in the presence of FGF2 *in vitro*,

Figure 3. (Opposing page) *Multipotent NSCs of the adult mouse forebrain.* The subventricular zone (SVZ) of the lateral ventricles of the adult mouse forebrain contains undifferentiated precursor cells that, when plated in the presence of FGF2 ((**A**), 2 DAP), become hypertrophic, proliferate ((**B**), 6 DAP) and, eventually, give rise to spherical clusters of undifferentiated cells ((**C**), 18 DAP). When spheres are grown adhered to a substrate in the absence of growth factors, these cells differentiate (**D**), generating the three major mature neural lineages. As shown in (**E**), neuronal (MAP2, **red**, **arrow**), astroglial (GFAP, **green**), and oligodendroglial (**blue**, **arrowhead**) markers can be detected within the differentiated progeny of adult FGF2-responsive NSCs by multiple immunofluorescence assays (see also ref. (37)).

hippocampal precursors (albeit of uncertain clonal relationships) have displayed the capacity to reintegrate into the host hippocampus following intracerebral grafting, without tumor formation (31). Strikingly, when the site of engraftment was switched to the rostral migratory pathway via which neuronal precursors in the forebrain SVZ reach the OB (118, 136), the hippocampal cells were able to integrate as normal olfactory neurons and to express tyrosine hydroxylase (TH), an enzyme not normally found in hippocampus. Interestingly, suggesting some element of restricted potential, the same cells failed to differentiate into neurons when engrafted into the cerebellum (31, 118). EGF-expanded mouse cells have been shown to engraft following implantation in the embryonic rat brain, although their differentiation repertoire *in vivo* appeared to be more restricted (to a glial phenotype) than one might have expected (138). That this type of engraftment may nevertheless have therapeutic utility was suggested by an experiment in which EGF-expanded cells derived from a transgenic mouse in which nerve growth factor (NGF) expression was driven by a GFAP promoter could attenuate excitotoxic striatal lesions (11).

The above-mentioned observations suggest that multipotent stem-like neural cells can be isolated from the rodent brain and reliably propagated by epigenetic means. While the full extent of the plasticity and therapeutic potential of such cells remains to be more extensively explored, if they continue to emulate the behavior of the more extensively studied CNS stem–like cells that have been isolated, cloned, and propagated by genetic means (discussed in the next section), then they should prove a promising addition to the armamentarium for NSC research and clinical applications.

Genetic means for the propagation of multipotent neural stem-like cells. External (i.e., epigenetic) growth stimulatory signals (such as cytokines) and the various genetic means for propagating cells all appear to converge on similar final common pathways that influence the expression of various cell cycle regulatory elements (16, 23, 35, 41, 79, 115). The key to optimally cloning, characterizing, manipulating, and utilizing NSCs is to suspend temporarily their differentiation — i.e., to block transiently and in a reversible manner their otherwise cascading progression through a developmental program — by prompting the cells to remain in the cell cycle in culture. With epigenetic stimulation, the mitogenic agents can be added or removed from the culture medium. Alternatively, one may insert genes whose products "automatically" interact in a constitutively self-regulated manner with cell cycle proteins; the transduction of such "propagation" genes into progenitor cells isolated from the developing CNS has proven to be a safe, effective, and convenient method for isolating and propagating neural stem-like cells (5, 29, 30). In this approach, as with epigenetic stimulation, the pressure to proceed through a particular developmental program is reversibly suspended *in vitro* as a result, most likely, of these genes acting in concert with conditions characteristic of the tissue culture milieu as well as the elimination of the differentiation cues normally found *in vivo*. Nevertheless, while maintaining NSCs in a proliferative state *in vitro* "suspends" their progression through developmental programs that usually entail a rapid narrowing of their phenotypic options, this holding of differentiation in abeyance neither "subverts" these developmental programs nor abrogates the ability of the NSCs to respond appropriately to normal environmental cues *in vivo*, to withdraw from the cell cycle, eventually to undergo terminal differentiation, and to interact with endogenous cells in a region-specific manner (110). Importantly, the cells appear to exit the cell cycle not in the tissue culture dish but in the brain where they are influenced, at that most vulnerable stage, by the microenvironment of the host CNS (an advantage over most epigenetic propogation techniques).

Different isoforms of myc, neu, p53, adenoviral E1A and SV40 large T-antigen (T-ag) have been used to propagate cells from different CNS regions and donor ages (e.g., 5, 12, 13, 29, 58, 61, 91, 96, 99, 133, 134; reviewed in 67a, 69, 135). The genes that have been used most extensively have been vmyc and a mutated allele of T-ag, the tsA58 temperature sensitive form of the protein. This latter gene product is stable at the permissive temperature (33°C) and induces cells to progress through the cell cycle, but is degraded when the temperature is raised to 37°C in culture or following grafting into the rodent brain where the temperature reaches 39°C. Notably, following their integration into the brain, neural cells transduced with vmyc, constitutively and spontaneously downregulate v*myc* immunoreactivity, suggesting loss of expression or degradation of the gene product (28, 114).

All of these clones behave like established, stable cell lines. They do not show signs of transformation either *in vitro* or *in vivo* (135). In culture, they become contact-inhibited and cannot grow in soft agar. In grafting studies, though the number of cells often seen at maturity suggests 0-3 mitoses prior to terminal differentiation after implantation, no brain tumors are ever seen; the donor-derived cells insinuate themselves seamlessly and

non-disruptively into the host cytoarchitecture (12, 61, 83, 114). Furthermore, the total number of cells (host plus donor) observed in a given engrafted region equals that observed in an analogous region of an untransplanted animal (i.e., host cells alone), suggesting that these donor NSCs do not abnormally augment or deform their region of integration, but rather compete equally for space with host progenitors. These various observations are in agreement with the finding that, when recipient animals are "pulsed" with bromodeoxyuridine (BrdU), the proportion of donor cells that are mitotic falls to 0 by 48-72 hours post engraftment, a phenomenon that mirrors their behavior in culture following contact inhibition (83, 114). Accordingly, transplanted mice exhibit no neurological dysfunction and CNS regions within which donor cells engraft seem to develop normally. In fact, recent preliminary studies have shown that they even function in concert with host cells in a physiologically-appropriate manner. For example, donor NSCs grafted to the developing suprachiasmatic nucleus of the hypothalamus, the light-sensitive center for circadian rhythmicity in the mouse, will appropriately upregulate c-fos in response to photic stimulation to the eyes in precisely the same manner as activated endogenous neurons of that region (143).

Genetically-propagated NSCs were among the first to be experimentally tested in neuroregeneration paradigms and, therefore, to suggest the ultimate promise of the stem cell for therapeutic intracerebral grafting. A number of characteristics confer particular advantage to such cells in these various gene transfer and cell replacement paradigms (to be discussed later): *i.* They are "self-renewing", permitting their indefinite expansion and passaging in culture; while the progeny of one cell may exit the cell cycle and differentiate, other progeny of that cell will replenish (and perhaps even enlarge) the stem cell pool by remaining immature, proliferative, and multipotent. *ii.* Their mitotic nature allows the ready introduction of reporter and therapeutic genes by most viral- and non-viral-mediated transduction strategies. *iii.* They have been isolated as single clones, making their genetic background homogeneous, in contrast to cultures of primary neural tissue or even of most reported growth factor-expanded progenitor populations. *iv.* They are of CNS-origin and can integrate quite facilely within a recipient host brain following transplantation. *v.* They remain consistently multipotent even over multiple passages, differentiating into glia and neurons *in vitro* and *in vivo*.

The most striking properties of these propagated NSC clones are the last two listed above, i.e., their

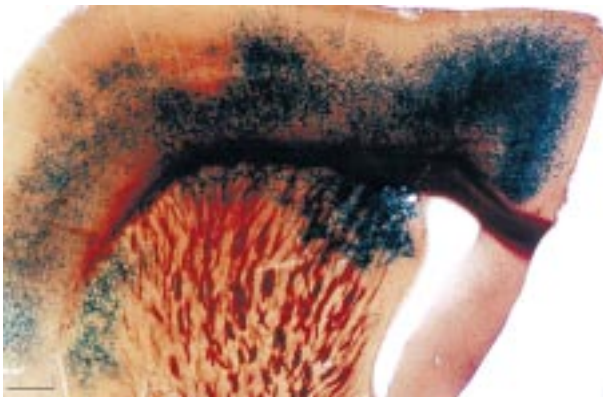

Figure 4. *Representative coronal section from a mature mouse brain that received a transplant of murine NSCs in midgestation.* A stable clone of murine NSCs (clone C17.2), engineered to express the *lacZ* reporter gene, was implanted into the ventricular germinal zone (VZ) of an embryonic mouse (embryonic day 12). At that time in development, the VZ gives rise to both neurons and glia throughout the CNS. At adulthood, the host brain was processed for *lacZ* expression by the Xgal histochemical reaction which yields a blue precipitate. Blue-staining cells, the descendants of the implanted NSCs, were integrated throughout the recipient brains. The NSCs differentiated into multiple cell types, both neuronal and glial, in many CNS regions along the neuraxis, responding to signals of the respective region at the particular developmental stage of the transplant. Certain cell types were apparent that are normally born only within the narrow developmental window of these transplants, e.g., pyramidal neurons in the middle lamina of the neocortex. (Reviewed in Ref. 114). Scale bars: 400 μm. (Reproduced with permission from Ref. 110.)

apparently seamless integration into the surrounding parenchyma of a recipient brain after transplantation (Figure 4) and their differentiation into ostensibly mature neural cells of various phenotypes. In contrast, for instance, to primary fetal neural tissue, fibroblasts, or muscle cells, NSC clones rapidly migrate from their implantation site, often leaving appropriate germinal zones, and travel well-established migratory pathways to eventually integrate in a cytoarchitecturally proper manner.

The above observations were initially made for the C17.2, C27.3, and HiB5 NSC clones grafted into the newborn brain in studies performed to analyze their developmental potential (91, 99). These cells were found to integrate well and to differentiate into both neurons and glia in the recipient brain, resembling the host cell types found in the site of engraftment, the hippocampus or cerebellum.

Later on, a series of striatum-derived cell lines (ST14A, ST79-13A, ST86) in addition to HiB5 cells and clone C17.2 NSCs were successfully grafted into

multiple CNS regions of fetal, neonatal, adult, and even aged recipients, displaying appropriate patterns of integration and differentiation (12, 49, 59, 61, 65, 100-114). Following transplantation, particularly into the cerebral ventricles, the cells slip unimpeded through the blood-brain barrier (BBB) and become integral members of the cytoarchitecture (110, 114) (Figure 4). Donor-derived neurons receive appropriate synapses and possess appropriate ion channels; the BBB remains intact where stem cell-derived astroglia put foot processes onto cerebral vasculature; donor-derived oligodendrocytes express myelin basic protein (MBP) and are capable of myelinating neuronal processes (63, 100, 110-113). Strikingly, such cells can also functionally integrate into host neural circuitry. This is suggested by the observation, as described above, that clone C17.2 NSCs, incorporated into the hypothalamic suprachiasmatic nucleus, cyclically upregulate their c-fos expression in response to circadian photic stimulation of the animal (143), and that HiB5 NSCs incorporated in the hippocampal dentate gyrus, overexpress c-fos in response to local experimentally-induced seizures (73). Interestingly, some donor-derived cells integrate as simply undifferentiated, quiescent progenitors or stem cells (12, 60, 61, 110-113) that often reside in tight association with blood vessels, resembling pericytes, or are positioned in the SVZ, or are even interspersed with other more differentiated cells within the CNS parenchyma. This observation is consistent with the interesting finding that a sub-population of clone C17.2 NSCs, stably engrafted at midgestation into the forebrain, could subsequently be recultured from the recipient at adulthood and could go on to display the typical broad developmental potential of that NSC clone when later reimplanted into entirely different regions (e.g., the cerebellum) of other animals and different developmental stages (100, 114). This experiment closely resembles the serial transplantation approach used to study stem cells in the hematopoietic system and strongly supports the idea that some NSCs retain their stem-like characteristics following intracerebral engraftment. It is also consistent with the finding that cells with stem-like features can be cultured from the parenchyma from various adult CNS regions (including the cortex) and propagated by mitogens (82). Present thinking holds that, in such cases, these growth factors (e.g., EGF, FGF2) are not causing mature cells to de-differentiate, but rather are forcing residual but quiescent stem cells (presumably deposited during initial organogenesis) back into the cell cycle.

What is the fate of a stem cell grafted in a given region? Donor stem cells (as exemplified by the prototypical NSC clone C17.2) appear to mirror the normal developmental processes ongoing in the region in which they integrate. In other words, they not only intermingle with local endogenous progenitors/stem cells in a non-disruptive fashion, but also respond to environmental cues in the same manner as host cells. Accordingly, while they have the *potential* to give rise to multiple and varied cell types, their *fate* is to differentiate only into the types of neurons and glia expected for a respective region at the particular developmental stage during which the transplant takes place. Thus, in regions where neurogenesis is ongoing, these cells give rise to the appropriate neuronal cell type; in regions where neurogenesis has already ceased, and where gliogenesis is the predominant process, they differentiate solely into glia. For instance, clonal NSCs differentiate into pyramidal neurons in neocortex when implanted into the ventricular zone (VZ) of a mid-embryonic mouse brain during normal corticogenesis, but do not undergo neuronal differentiation in the same area when, at a later stage, neurogenesis is completed and gliogenesis takes place (58, 107, 110-114). The same clone of NSCs can give rise to Purkinje cell (PC) neurons in the cerebellum of embryonic day 12-14 (E12-14) mouse (when PCs are born); at a later stage, however, they generate only the cell types normally born then, i.e., small interneurons and glia. In the adult brain, most neurogenesis has ceased with the exception of that in the hippocampus and in the OB. Endogenous progenitors in the SVZ will migrate to the OB and become granule neurons. Accordingly, when the above-mentioned clone of NSCs are implanted into the ventricles of an adult mouse, they intermingle with host SVZ cells and migrate with them to the OB via the rostral migratory stream where they differentiate into granule neurons. Cell from that same NSC clone that happen to migrate from the adult ventricles into the neocortex (or into most other regions of the adult brain, where gliogenesis predominates), produce exclusively glia (103-106, 114). This, of course, is the very same clone of NSCs that, if similarly implanted in the ventricles of a *fetus*, can still give rise to the aforementioned pyramidal neurons. Thus, for research purposes, stable, well-established NSC clones (such as those generated by genetic means) provide a useful tool for probing and accurately reflecting the extant developmental processes within given spatial and temporal windows.

Survival and integration of NSCs following transplantation appears to be stable and lifelong. Donor cells do not seem to induce host-mediated immune rejection despite their various genetic modifications. Donor-derived cells have been identified in transplanted ani-

mals for at least 2 years after grafting with no marked reduction in number (61, 99). (That NSCs are so well tolerated immunologically when transplanted within their species of origin may derive from the interesting observation that, in their undifferentiated state, NSCs do not appear to express significant amounts of MHC class 1 or 2 on their surface (110).)

Neural Stem Cells as a Therapeutic Tool

Regardless of the method used for their isolation, propagation, and amplification in culture, stem-like neural cells likely represent valuable therapeutic tools against a variety of neuropathologies. Their clinical potential is rooted in their inherent biologic properties. Their ability to develop into integral cytoarchitectural components of many regions throughout the host brain as neurons, oligodendrocytes, or astrocytes, makes them capable of replacing a range of missing or dysfunctional neural cells. NSCs may also be used as cellular vectors for the stable *in vivo* expression of foreign genes of developmental and/or therapeutic relevance (3, 49, 63-66, 99-104) that can be delivered, if necessary, throughout the host CNS (49, 63, 100, 103, 109). In fact, these cells are amenable to various types of viral vector transduction (3, 40, 49, 64-66, 96-99, 104, 109) as well as to other strategies of transfection, such as lipofection, electroporation, and calcium-phosphate precipitation. Since they display significant migratory capacity as well as an ability to integrate widely throughout the brain when implanted into germinal zones, NSCs may help reconstitute enzyme and cellular deficiencies in a global fashion (109). Their ability to accommodate to their area of engraftment probably obviates the need for using stem cells from specific CNS regions. Furthermore, NSCs appear to possess a tropism for and trophism within degenerating CNS regions (27, 84, 99), actually modifying their differentiation fate to replenish specific deficient neural cell pools. This fact fosters the view that some neurodegenerative processes elaborate neurogenic signals that recapitulate developmental cues to which NSCs can respond.

These attributes of NSCs may provide multiple strategies against CNS dysfunction. Some of these approaches have already shown promise experimentally in mouse models of neurodegeneration (101-114). Some illustrative examples are briefly discussed below.

Neural Stem Cells for Gene Transfer. The ability of NSCs to deliver therapeutic gene products in an immediate, direct, sustained, and, perhaps, regulated fashion as normal cytoarchitectural components throughout the CNS may overcome some of the limitations of standard viral and cellular vectors (reviewed in (108, 109, 112, 119)). The feasibility of this strategy was initially demonstrated in the murine model of the lysosomal storage disease mucopolysaccharidosis type VII (MPS VII), a disease linked to the deletion mutation of the β-*glucuronidase* (GUSB) gene. This inheritable condition is characterized by neurodegeneration in mice and by progressive mental retardation in humans (104). GUSB-secreting NSCs were implanted into the cerebral ventricles at birth, allowing them access to a germinal zone from which they were disseminated throughout the brain, allowing them to correct lysosomal storage throughout the brains of mutants in a permanent manner. This therapeutic approach is now being extended to animal models of other untreatable neurodegenerative diseases. Retrovirally-transduced NSCs, implanted into the brains of fetal and neonatal mice, have successfully mediated widespread expression throughout the brain of the β-subunit of β-*hexosaminadase*, a mutation of which leads to the pathological accumulation of GM2 ganglioside, the abnormality in Tay-Sachs disease (49). Findings such as these have helped to establish the paradigm of using NSCs for the transfer of other factors of therapeutic or developmental interest into and throughout the CNS (140). To date, NSCs have been used for the delivery of NT-3 within the hemisectioned rat spinal cord (40, 54a) and asphyxiated brain (84), to express NGF and BDNF within the septum and basal ganglia (64-66, 102), tyrosine hydroxylase in the striatum (3), Reelin in the cerebellum (4), and MBP throughout the cerebrum (139).

Neural Stem Cells In Models of Abnormal Development. Knowing that many neurological diseases are often characterized by *global* cell degeneration or dysfunction, rodent NSCs have been tested for their ability to address this type of pathology, not traditionally viewed as amenable to neural transplantation. It was hypothesized that the intraventricular injection technique employed for the dissemination of enzyme-producing NSCs to achieve *gene product* replacement might be applied as well to achieve widespread neural *cell* replacement. Mouse mutants characterized by CNS-wide white matter disease were used to test this hypothesis. The oligodendroglia of the dysmyelinated *shiverer (shi)* mouse lack MBP and are, therefore, incapable of effective myelination. Effective therapy requires extensive replacement with MBP-expressing oligodendrocytes. NSCs transplanted at birth into the *shi* brain could, indeed, produced widespread engraftment and repletion of significant amounts of MBP throughout the *shi* brain.

Furthermore, a subgroup of the donor-derived oligodendroglia myelinated on average 40% of host neuronal processes (139) in engrafted regions; some recipients, in fact, experienced a decrement in their symptomatic tremor.

Work in the *reeler* mutant mouse suggested that NSCs may also help partially to correct abnormal cytoarchitecture in models of dysfunctional lamination characterized by deficiencies in extracellular matrix (ECM) (4). The assignment of laminar position of neurons in the *reeler* brain is abnormal due to a mutation in the Reelin-encoding gene. NSCs implanted at birth into the external germinal layer of the developing *reeler* cerebellum promoted a more normally-laminated cytoarchitectural appearance in engrafted regions: not only did donor-derived granule neurons migrate to the appropriate layer, but they appeared capable of influencing neighboring mutant neurons, as well. *Reeler* PCs were more appropriately aligned in engrafted regions and the survival and deeper positioning of *reeler* GCs in the internal granular layer was also enhanced. Thus, donor NSCs may promote normal layering and survival by providing molecules (including Reelin) that can work at the cell surface to guide proper migration and positioning of neurons during histogenesis. These findings, therefore, suggest a possible NSC-based strategy for the treatment of CNS diseases characterized by abnormal cellular migration, lamination, and cytoarchitectural arrangement.

Responsiveness of NSCs to CNS injury. It would be appealing, indeed, if *neural* stem cells possessed the capacity to respond to CNS injury in a manner analogous to the response of *hematopoietic* stem cells to *bone marrow* ablation. Recent studies have attempted to assess the behavior of clonal NSCs in response to cues found in the microenvironment of the injured CNS. Because of the extent of damage rendered by hypoxic-ischemic (HI) CNS injury (about as analogous to "bone marrow ablation" in the brain as neurobiologists are likely to come), and because HI patterns a genuine clinical condition in humans, this model has been recently employed in pilot studies. Two complementary strategies of investigation have been used: (1) Normal, intact mice were engrafted at birth with clonal NSCs *prior* to HI, allowing the cells to become normal components of the brain (indeed, creating chimeric regions of brain). (2) The same clone was implanted at various intervals *following* HI. The clone of NSCs employed expressed the reporter gene *lacZ constitutively* and stably; hence the behavior of NSCs and their clonally-related progeny could be traced and assessed by virtue of their *lacZ* expression (83, 84a). Unilateral HI was induced throughout one hemisphere by ligation of the ipsilateral common carotid artery in week old mice followed by exposure to insufficient oxygen. This model renders extensive degeneration throughout the affected hemisphere while leaving the contralateral hemisphere as an intact control.

In the first paradigm, in response to HI, a subset of quiescent NSCs (a) transiently re-entered the cell cycle within 24 hours from the HI, undergoing cell division for 3-4 days and then returning to quiescence; (b) migrated to the site of injury; and (c) differentiated into neurons and oligodendrocytes, the neural cell types most damaged following HI. These behaviors are precisely those expected from a quiescent stem cell pool trying to repopulate a damaged region in response to injury (55, 84a).

In the second paradigm, in which NSCs were implanted at various time points following HI into or near the ischemic area, or into distant locations, donor NSCs integrated extensively within the infarcted areas that spanned the length of the brain. Grafted NSCs in these pilot studies migrated from even distant locations towards the lesioned areas. Again, a subpopulation of donor NSCs differentiated into neurons and oligodendrocytes, as if attempting to repopulate the area of injury. Interestingly, if transplantation was performed 5 weeks after HI, engraftment was negligible, suggesting a "permissive window" for this phenomenon.

It is noteworthy to recall that neurogenesis in the non-fetal, "post-developmental", intact neocortex does not occur. And, indeed, consistent with this classic dogma, no donor-derived neurons were detected in the cortex of the intact hemisphere. Nevertheless, in response to and within a narrow temporal window following injury, new neurogenesis derived from NSCs *could* be observed even in CNS regions classically characterized as "non-neurogenic" at that developmental stage.

Notably, in preliminary studies, when subclones of NSCs, genetically engineered *ex vivo* to overexpress NT-3, are implanted into the asphyxiated mouse brain, the percentage of donor-derived neurons increases 16-fold and new host-derived neurons are also detectable, suggesting that NSCs may play a unique therapeutic role by mediating both cell replacement and gene therapy simultaneously within the same animal during the same grafting procedure. (84, 84a).

The idea that developmental signals may be re-expressed during phases of active degeneration to which

NSCs may be responsive is reinforced by preliminary experiments conducted in a model of spinal lower motor neuron (LMN) apoptosis. In this system, as well, it appears that donor NSCs might be "shifting" their differentiation fate specifically towards replacement of dying LMNs. NSCs implanted into the L5 ventral horn 2-4 weeks after neonatal sciatic axotomy differentiate into cells that appear to be "replacing" some of the degenerated LMNs. The number of engrafted NSCs that assume an LMN phenotype (a cell type not normally born at that developmental epoch) is tripled when transplantation is performed closer and closer to the peak of active LMN apoptosis. Indeed, in the midst of this more actively degenerating *milieu*, NSCs can be implanted into even the *dorsal* horn from which they migrate to the region of LMN loss in the *ventral* horn. Engrafted donor-derived cells continue to express *lacZ*, suggesting that the implantation of genetically-engineered, neurotrophin-expressing NSCs might enhance neuronal differentiation, neurite outgrowth, and proper connectivity in this model of CNS injury as they do for the HI injury described above.

Human CNS Stem Cells

Our knowledge regarding the functional properties and therapeutic potential of stem-like neural cells is essentially based on studies conducted on cells derived from the embryonic, neonatal, or adult *rodent* CNS. The expectation, however, — indeed, the leap of faith — is that the basic physiology that endows rodent stem-like cells with their therapeutic potential is conserved in cells of *human* origin.

In fact, evidence is quite scarce concerning the existence in humans of undifferentiated, multipotent neural progenitors that are capable of differentiation into neurons and all other neural cell types. The presence of mitotically active neuronal precursors in the human hippocampal dentate gyrus — analogous to those long known to exist in the rodent hippocampus — has recently been observed *in vivo* (24). Neuronal precursors capable of mitotic activity (78) have been isolated from the cortical, subcortical, and periventricular (subependymal) regions of the adult human temporal lobe, although their full lineage capacity and the extent of their proliferation potential remains to be determined (47, 86). Until recently, the identification, isolation, characterization, and propagation of stable clones of self-renewing, multipotent human NSCs, similar to the rodent cells described above, remained somewhat elusive. In fact, when the widely used epigenetic approach for expanding rodent stem-like cells in the presence of EGF in serum-free medium was employed for propagating CNS cells of human origin, no significant growth could be achieved (14). It was only by combining EGF with insulin-like growth factor-1 (IGF1) that proliferation of neuronal precursors from the fetal human cortex, striatum, and basal forebrain could be accomplished. Although these cells could be differentiated into catecholaminergic, GABA'ergic, cholinergic, and peptidergic (i.e. substance P-expressing) neurons, this culture system still did not allow for extensive cell growth or clonal analysis. Thus the lineage capacity of these human precursors remained unclear. Furthermore, the finding that cell growth declined over just a few passages suggested that they lacked the capacity for self-renewal and were best described as transiently dividing neural progenitors, a cell type of limited proliferative capacity known to appear transiently in various tissues during embryonic development (55). Similarly, the use of EGF, either alone or in combination with FGF2, yielded extremely limited, if any, cell growth and amplification during attempts at isolating stem-like cells from the CNS of 13- or 22-week old human fetuses (120, 121). In fact, until quite recently, the only renewable source of human neurons was represented by immortalized bipotent embryonic precursors (98) or teratocarcinoma-derived cells, successful transplantation in various experimental settings having been demonstrated only for the latter (123). Within the past few months, however, the isolation and propagation of engraftable human CNS stem cells has been achieved (28, 128) and is reviewed in the following sections.

Cloning, expansion, propagation, & differentiation of bona fide human CNS stem cells. According to the operational definition discussed previously, an assessment of the functional attributes that are critical for the unequivocal identification of a stem cell must be conducted at the single cell level, i.e., in a clonal fashion. Our two groups have tackled the issue of isolation, propagation, and cloning of NSCs from the developing human CNS by means of complementary epigenetic and genetic methods, reaching very similar results (28, 128). The strategies used to isolate human NSCs mirrored those used for the very tractable and therapeutically promising murine NSC clone C17.2 (i.e., transduction of *lacZ* and/or the constitutively downregulated v*myc*) (114) and those for the widely emulated growth factor-expanded murine NSCs (31, 47a, 126, 131). Strikingly, both genetically and epigenetically propagated cells (whether of murine or human origin) require molecules like FGF2 and/or EGF to proliferate in serum-free conditions (28, 47a, 110, 111).

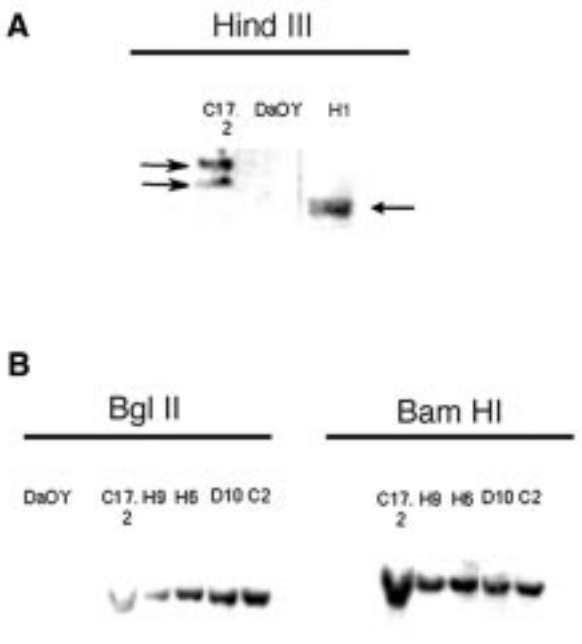

Figure 5. (Left) *The monoclonal nature of each putative human NSC clone is confirmed by demonstrating a single retroviral insertion site within the genomes of each.* An NSC is defined as a single cell which is capable of giving rise (*in vitro* and/or *in vivo*) to neurons (of multiple types), oligodendrocytes, and astrocytes, as well as new NSCs. That these progeny are clonally-related to that single cell and to each other is an obligatory part of the definition. Therefore, an unambiguous demonstration of monoclonality is a *sine qua non* for affirming both multipotency and self-renewal. (**A**) Genomic DNA from the putative human NSC clone H1 (which was propagated in bFGF and subsequently transduced with a retrovirus encoding *lacZ* and *neo*) was digested with Hind III (which cuts only once within the provirus) and incubated with a radiolabeled nucleotide probe complementary to *neo*. Monoclonal derivation is confirmed by the presence of a single integrated retrovirus with an integration site common to all cells in the colony indicating that they were derived from a single infected "parent" cell (**arrow**). As a positive control, the murine NSC clone C17.2 which contains 2 integrated proviruses encoding *neo* (one from an integrated v*myc*-encoding retrovirus and one from a separate *lacZ*-encoding retrovirus (96, 99) appropriately shows 2 bands (**arrows**). Specificity of the probe is demonstrated by the negative control, the human meduloblastoma cell line DaOY, which, having not been infected with a retrovirus, shows no *neo* sequences in its genome and hence no hybridization product. (**B**) Genomic DNA from putative clones H9, H6, D10, and C2 (human NSC colonies propagated in bFGF and/or EGF and then subsequently infected with a retrovirus encoding the propagating gene vmyc) were digested with Bgl II or Bam HI (each of which cuts only once within the provirus) and then subjected to Southern analysis utilizing a probe complementary to the proviral v*myc*. Single retroviral integration sites are appreciated in all colonies confirming the monoclonal nature of each putative clone. The murine NSC clone C17.2, which contains a single copy of v*myc* (96, 99) and serves as a positive control, also has one band. As in (**A**), the negative control non-virally infected human DaOY cells, have no bands. (Reproduced with permission from Ref. 28)

In one approach (28), cultures were established from the VZ of a 15 week human fetus and were enriched by growing them in serum-containing medium first, followed by a switch to and selection by serum-free medium supplemented with saturating concentrations of EGF and/or FGF2. Responsiveness to *both* factors (based on experience with murine stem-like cells (37, 47a, 131)) was regarded as a pivotal screen for the most immature, primordial, uncommitted, and multipotent of neural cells. Cells whose well-being and passageability could not successfully tolerate being switched from serum-free medium containing first one growth factor and then the other and then back again would not survive, leaving a population of healthy cells that presumably bore functional receptors for both (a characteristic shared with clone C17.2 murine NSCs). Various populations that survived this first screening and enrichment process were then screened for "engraftability": they were transplanted into the ventricles of newborn mice; populations that could not yield engraftable cells that would integrate and migrate in the developing brain were also no longer maintained. Finally, those engraftable populations were screened for the presence of neural cells in all lineages by immunocytochemical criteria *in vitro*. Ultimately, this multi-stepped screening process yielded a polyclonal population of EGF and FGF2 dual-responsive, engraftable cells of multiple lineages that could then be used for further manipulation, cloning, and characterization at the single cell level. Some populations were 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 FGF2-propagated subpopulations were infected with an amphotropic replication-incompetent retroviral vector encoding *lacZ* (and the neomycin-resistance gene *neo* for selection). Single neomycin-resistant colonies were initially isolated by limiting dilution; monoclonality of the cells in a given colony was then confirmed by demonstrating that only 1 copy of the *lacZ/neo*-encoding provirus, with a unique chromosomal insertion site, was present. In clone "H1", for example, all *lacZ/neo+* cells, indeed, had a single, common retroviral integration site indicating that they

Brain Region	Embryonic Age	Embryo	Highest Passage Number	Average Doubling Time
Presumptive Diencephalon	10.5 weeks	Normal	54	7-10 days
Presumptive Diencephalon	10 weeks	Normal	34	12-15 days
Cortex	10 weeks	Normal	32	12-14 days
Cortex	6 weeks	Normal	30	10-12 days
Telencephalon	10.5 weeks	Normal	40	8-10 days
Lumbar Spinal Cord	12 weeks	Normal	32	15-20 days
Whole Brain	9.5 weeks	Normal	26	7-10 days
Brainstem	14 weeks	Olivo-Ponto-Cerebellar Atrophy (SCA1)	38	5-7 days
Cortex	14 weeks	Olivo-Ponto-Cerebellar Atrophy (SCA1)	25	8-10 days
Cerebellum	14 weeks	Olivo-Ponto-Cerebellar Atrophy (SCA1)	10	9-12 days
Whole Brain	14.5 weeks	Friederich's Ataxia	36	5-7 days
Cortex	14 weeks	Down's Syndrome	28	10-12 days
Brainstem	14 weeks	Down's Syndrome	24	8-10 days
Whole brain	16 weeks	Down's Syndrome	32	7-9 days
Whole brain	19 weeks	Down's Syndrome	20	10-12 days
Lumbar spinal cord	14 weeks	Huntington disease	14	12-15 days
Cortex	13 weeks	Trisomy 13	15	10-12 days
Diencephalon	13 weeks	Trisomy 13	18	8-10 days
Cortex	18 weeks	Trisomy 18	13	8-10 days
Whole brain	18 weeks	Trisomy 18	18	8-10 days

Table 1. A representative list of neural stem cell lines established from various regions of the embryonic human brain.

were derived from a single infected "parent" cell (Figure 5A). As indicated above, because genetically-mediated isolation and propagation of rodent CNS material had yielded such safe, engraftable NSC clones that were not only easy to manipulate and utilize but that also had notable therapeutic power (114), some of the FGF2-maintained human cell populations, enriched for NSCs as described above, were infected with a retroviral vector encoding v*myc* and *neo*. Isolated drug-resistant colonies resulted. Again, monoclonality of each colony was affirmed by demonstrating that each putative clone had only 1 unique retroviral insertion site (Figure 5B). Thus, as was the case for the epigenetically-maintained cells, all v*myc*-transduced colonies were monoclonal. Five clones (H6, H9, D10, C2, E11) were generated and maintained in serum-free medium containing FGF2.

The multipotentiality of all of these various clones was then assessed by *in vitro* differentiation in which neurons, oligodendrocytes and astrocytes were observed under various differentiation conditions (see below). More importantly, these differentiated cultures still contained cells expressing vimentin (an immature neural marker) that were capable of further subcloning, following which they could again give rise to the 3 major neural lineages as well as to new vimentin-positive, passageable engraftable cells. Thus, the progeny of a single human neural progenitor was affirmed to possess both extensive self-renewing ability and multipotentiality.

The above findings found confirmation in a second complementary approach whereby cells from various regions and ages of the human embryonic brain (Table 1) were cultured immediately in serum-free medium containing both EGF and FGF2 upon dissociation from the primary tissue. From these cultures, which consistently expanded with a doubling time of 7-12 days (depending on their tissue of origin), single cells were plated in isolation (i.e. 1 cell/well) in the same growth medium. Time-lapse photography showed that individual, isolated cells gave rise to spherical clusters (Figure 6) that could be serially passaged to generate a continuous clonal line. As in the case of human neural cells expanded by genetic means, epigenetically-proliferated stem-like cells could be differentiated into the 3 major neural cell types (Figure 6) and could undergo self-renewal. In fact, by serial subcloning, it was shown that one multipotent cell could generate an average of 12 ± 4 secondary clone-forming cells at each passage; these each retained both multipotentiality and self-renewal capacity. The capacity for self-renewal was further confirmed in population studies of growth factor-expanded human stem-like cells which were predicated on the assumption that, if a cell is self-renewing (i.e. capable of producing identical copies of itself), then its various functional characteristics (e.g., growth rate and differentiation potential) should remain unchanged in its progeny even over prolonged serial propagation in culture. Consistent with this assumption, not only could human neural stem-like cells be exponentially expanded for

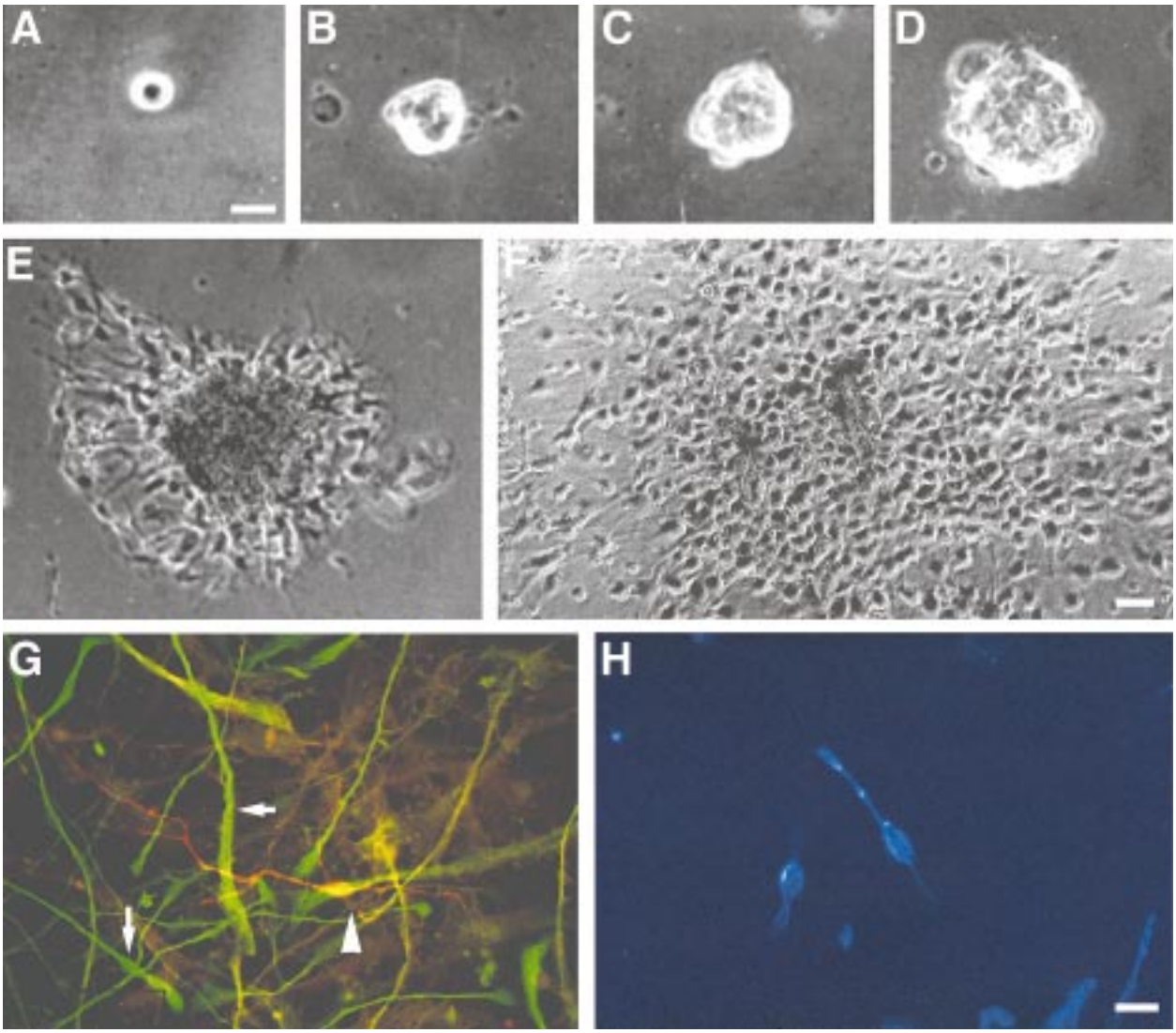

Figure 6. *Cloning of multipotential human CNS stem cells that generate neurons, astrocytes, and oligodendrocytes.* A single human diencephalic cell (10.5 week post-conception) is shown 1 day after plating in isolation (1 cell/well) in serum-free medium supplemented with both EGF and FGF2 (**A**). This cell proliferated ((**B**), 7 DAP; (**C**), 11 DAP) and, by 21 DAP, gave rise to a spherical clone (**D**). Single clonal clusters were grown further and then subcultured to generate secondary and tertiary clusters. The progeny of a single cell was plated onto separate glass coverslips (**E**) and allowed to expand (**F**). Differentiation was induced by the removal of growth factors, and cultures were processed for immunofluorescence assessment. Concurrent multiple immunolabeling shows the presence of neuronal (b-tubulin) ((**G**), **arrowheads**, **red**) and astroglial cells (GFAP) ((**G**), **arrows**, **green**) within a single cluster. Cells within another cluster from the same founder cell were identified as oligodendrocytes (O4) (**G**). Thus, multipotential, self-renewing NSCs can be isolated from the developing human CNS and expanded by epigenetic means (see also ref. (128)). Scale bars: (**A**)-(**E**), 20μm; (**F**), 18 μm; (**G**), 20μm.

over 2 years upon epigenetic stimulation, but, over this prolonged period, the first division always occurred after 3-4 days *in vitro* (DIV) after plating regardless of whether single cells were from early (10^{th}) or late (34^{th}) passages. In other words, the overall growth characteristics remained remarkably stable at the population level (Figure 7A). Similarly, their differentiation capacity, particularly their ability to generate neurons (Figure 7B), remained unchanged not only after serial sub-culturing, but also following repeated cycles of freeze-thawing. In addition, neurons prepared from long-term stem cell cultures were shown to express the ion channels necessary for excitability and were electrophysiologically active. In fact, neurons derived from passage

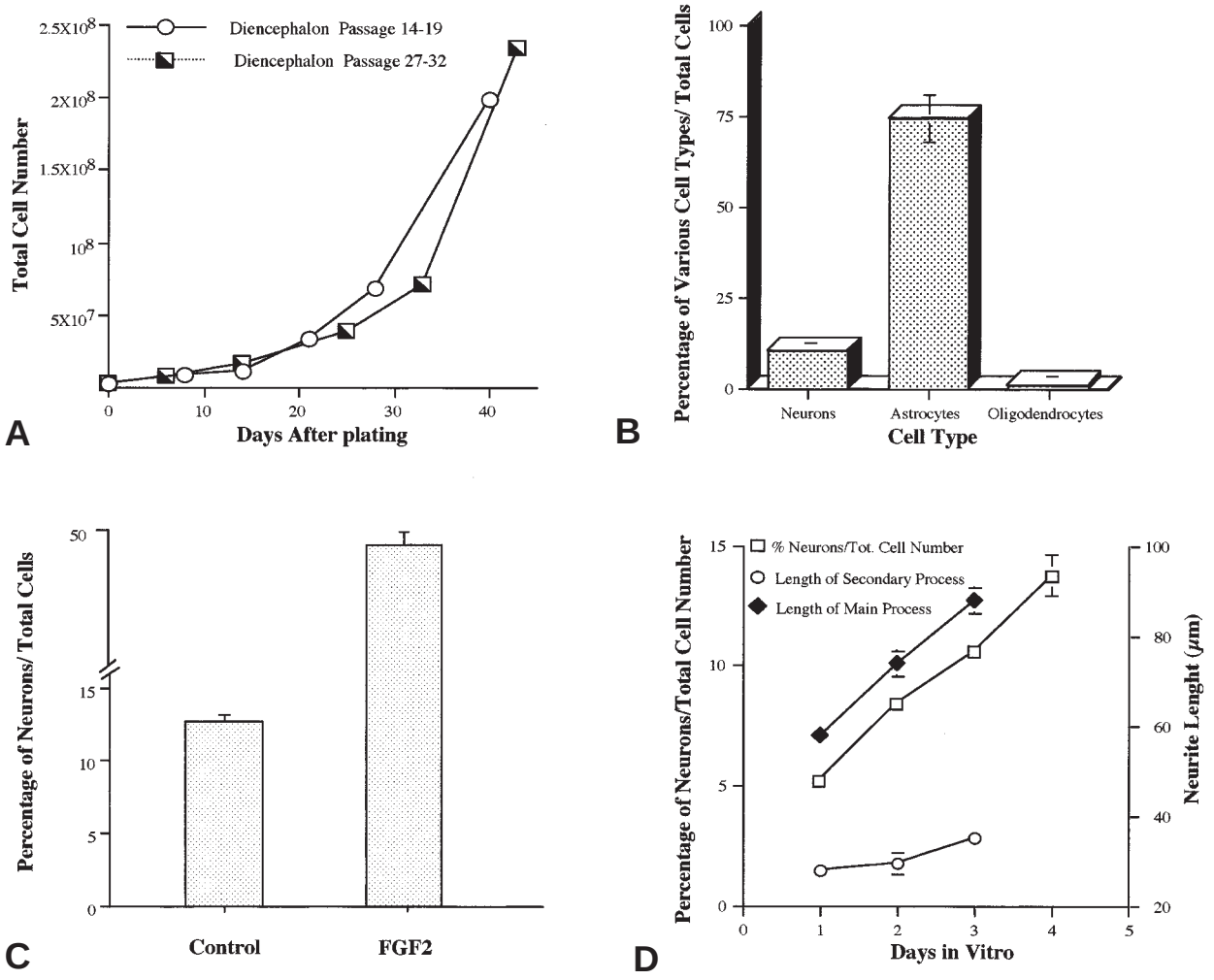

Figure 7. *Growth and differentiation of human CNS stem-like cells in culture.* (**A**) Human diencephalic stem cells from early (passage 14-19) and late (passage 27-32) cultures display comparable growth rates. These stem cells have now been steadily subcultured and expanded for over two years. Similar to their murine counterparts, human diencephalic stem cells can spontaneously differentiate into neurons, astrocytes, and oligodendrocytes (**B**) upon growth factor removal, irrespective of whether they are from early or late passages (data from 2 experiments; mean ± SE, n = 10). Importantly, the number of neurons generated by human CNS stem cells can be increased by up to 4-fold by inducing cell differentiation in the presence of low concentrations of FGF2 (**C**) (see also ref. (128)) as previously shown for mouse stem cells (88). (**D**) Neuronal differentiation/maturation occurs gradually following removal of growth factors, so that the number of neurons increases linearly and parallels the concomitant extension of neuronal processes, as detected by β-tubulin immunoreactivity.

34 stem cells generated single spikes in response to a brief current pulse and voltage-clamp experiments indicated the presence of the full complement of ionic channels necessary for the firing of self-sustaining action potentials.

One possible distinction between human NSCs expanded by epigenetic vs. genetic methods is the difference in their capacity to give rise to neurons. It is presently unclear, however, whether the observation that cells expanded via v*myc* generate approximately 5-6 times more neurons than those expanded by epigenetic means relates to the different regions of origin of the cells or is a reflection of the different methods used for their propagation (see also below). Nevertheless, a 4-fold increase in the number of neurons generated by growth factor-expanded NSCs can be achieved by modifying the differentiation conditions in culture (Figure 7C). Indeed, in cultures of both preparations of NSCs, neuronal differentiation and maturation initiates promptly following growth factor removal (and/or expo-

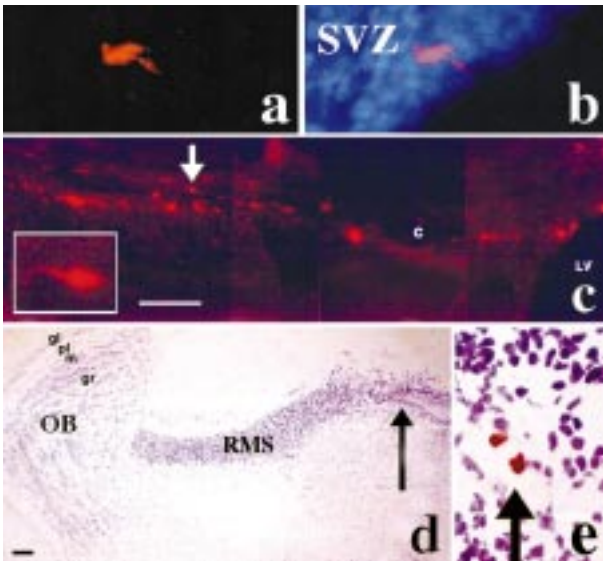

Figure 8. (Above, left) *Developmentally-appropriate migration of human NSCs following engraftment into the subventricular germinal zone (SVZ) of newborn mice.* (**A**, **B**) Donor-derived human NSCs integrate and intermingle nondisruptively with endogenous progenitors within the host SVZ by 24 hours after transplantation. A representative donor-derived cell with a typical short process (**red**), highlighted in (**A**), has interspersed with densely packed endogenous SVZ cells, visualized by DAPI (**blue**) in the overlapping image in (**B**). (**C**) Two weeks following transplantation, many donor-derived cells (**red**) have migrated extensively within the subcortical white matter (arrow) and corpus callosum (**c**) from their site of implantation in the lateral ventricles (**LV**), as visualized in this coronal section. A representative migrating cell within the subcortical white matter (**arrow**), visualized at higher magnification in the **boxed insert**, is noted to have a leading process characteristic of migrating precursor cells. (**D**, **E**) As seen in this representative cresyl violet-counterstained parasagittal section, other donor-derived cells migrated from their integration site in the anterior SVZ to enter the rostral migratory stream ("**RMS**") leading to the olfactory bulb ("**OB**"). Representative BrdU-immunoperoxidase-positive (**brown**) donor-derived cells (**arrow**) within the RMS, are seen at low power in (**D**) and visualized at higher magnification in (**E**), intermixed with migrating host cells. Further characterization of these human NSC-derived cells in their final location in the OB are presented in **Figure 9**. Layers of the OB within which these cells are visualized at higher magnification in **Figure 9** are indicated here: glomerular layer ("**gl**"), plexiform layer ("**pl**"), mitral layer ("**m**"), granule layer ("**gr**"). Scale Bars: 100μm. (Reproduced with permission from Ref. 28)

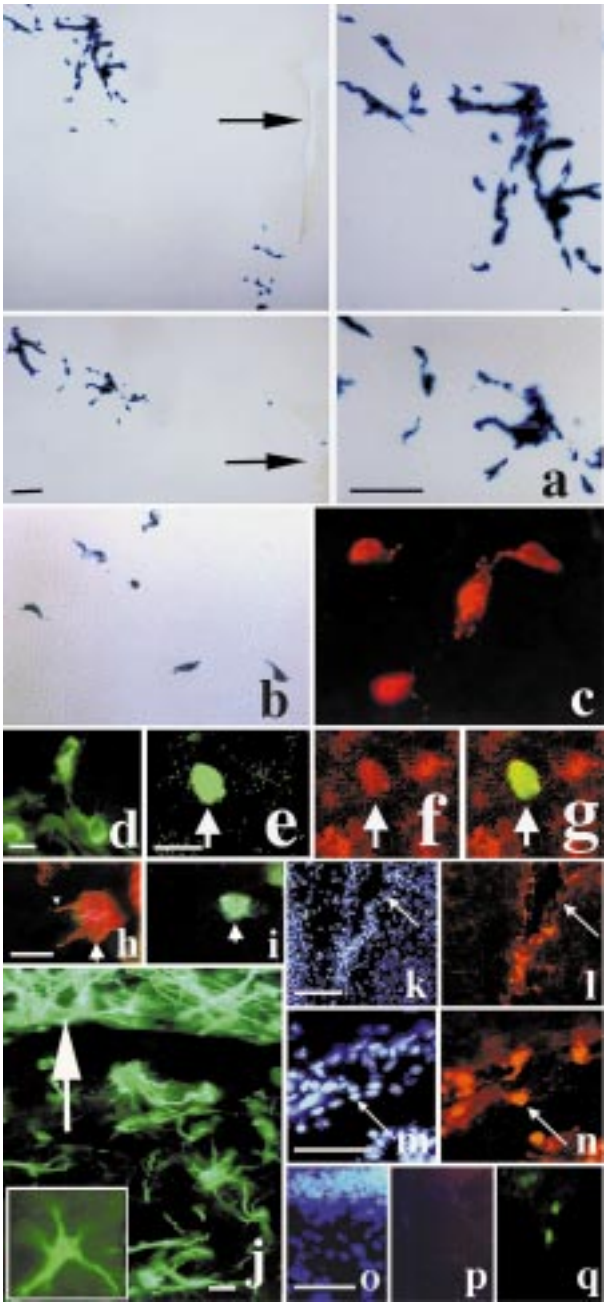

Figure 9. (Above, right) *Differentiation and disseminated foreign gene (β-galactosidase) expression of human NSC clones in vivo following engraftment into the SVZ of developing, neonatal mice.* (**A-C**) Stably engrafted, *lacZ*-expressing (β-galactosidase (βgal)-producing), donor-derived cells from representative human NSC clone H1, detected with Xgal histochemistry (**A**, **B**) and with anti-βgal ICC (**C**). The donor-derived cells pictured in the series of photomicrographs in (**A**) are within the periventricular and subcortical white matter regions (as per **Figure 8**). (The **top** and **bottom panels** — low power on the **left**, corresponding high power on the **right** — are from representative semi-adjacent regions within a single recipient, suggesting a significant distribution of cells; **arrows** indicate the lateral ventricles). Furthermore, as illustrated in (**B**, **C**) by representative high power photomicrographs through the olfactory bulb (OB) (granule layer ("**gr**") as delineated in **Figure 8D**), donor-derived cells from this clone have not only migrated extensively to this developmentally-appropriate site, but continue to express βgal in this distant location (i.e., in a disseminated fashion *in vivo*).

The normal fate of a subpopulation of SVZ-derived progenitors that have migrated to the OB at this developmental stage is to become neuronal. In (**D-G**), donor-derived neurons in the mature OB, derived from BrdU-labeled NSCs (representative clone H6) implanted into the SVZ at birth, are identified by both their immunoreactivity to a human-specific NF antibody (**D**) as well as their expression of the mature neuronal marker, NeuN (**E-G**); under confocal microscopy, a BrdU+ (hence, donor-derived) cell (**arrow** in (**E**), fluorescein) is NeuN+ (**arrow** in (**F**), Texas Red) appreciated best with a dual filter (**arrow** in (**G**)). Adjacent to this representative donor-derived BrdU+/NeuN+ neuron (**arrow**), are 2 host OB neurons (BrdU–/NeuN+ in (G)) which share a similar size, morphology, and location with the donor-derived cell (**arrow** in **F**). (Images in (**D-G**) are from the granule layer ("**gr**") as delineated in **Figure 8D**.) (**H, I**) High power view of a representative donor-derived (clone H6) oligodendrocyte (**arrow**), appropriately in the adult subcortical white matter (as per **Figure 8C**) following neonatal intraventricular implantation, double-labeled with an antibody to the oligodendrocyte-specific protein CNPase (**H**) and BrdU (**I**). Characteristic cytoplasmic processes extending from the soma are noted (**arrowhead** in (**H**)). (The morphology of the CNPase+ cell has been somewhat damaged by the HCl pretreatment required for BrdU double-labeling). (**J**) Mature donor-derived astrocytes (clone H6) in the adult subcortical white matter (**arrow**) (as per **Figure 8C**) and striatum following neonatal intraventricular implantation, identified with a human-specific anti-GFAP antibody. The **inset** better illustrates at higher magnification the characteristic mature astrocytic morphology of a representative human-GFAP+ cell. (**K-Q**) Expression of v*myc* is downregulated within 48 hours following engraftment. (**K**), (**M**), and (**O**) are DAPI-based nuclear stains of the adjacent panels (**L**), (**N**), and (**P**, **Q**), respectively. Representative human NSC clone H6 was generated (as was the well-characterized murine NSC clone C17.2) with the propagating gene v*myc*. vmyc immunoreactivity in H6-derived cells (**red**) in the SVZ (**arrows**) at 24 hours following engraftment ((**L**) and at higher power in (**N**)), is persistently absent (**P**) in integrated H6-derived cells (visualized by BrdU labeling in (**Q**) (shown here 3 weeks following transplantation, but representative of any point 24 hours after engraftment). Scale Bars: (**A**), (**K**) and applies to (**L**): 100μm; (**D**), (**E**) and applies to (**F**, **G**), (**H**) and applies to (**I**), (**J**), (**M**) and applies to (**N**): 10μm; (**O**) and applies to (**P**, **Q**): 50μm (Reproduced with permission from Ref. 28)

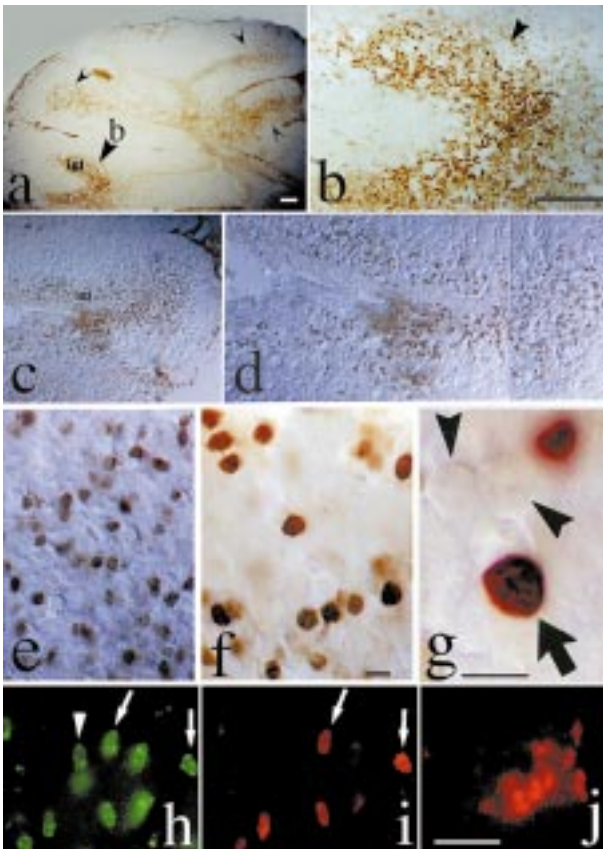

Figure 10. (Above, right) *Neuronal replacement by human neural stem cells (NSCs) following transplantation into the cerebellum of the granule neuron-deficient meander tail (mea) mouse model of neurodegeneration.* (**A-G**) BrdU-intercalated, donor-derived cells (from representative clone H6) identified in the mature cerebellum by anti-BrdU immunoperoxidase cytochemistry (**brown nuclei**) following implantation into the neonatal *mea* external germinal layer (EGL). (The EGL, on the cerebellar surface, disappears as the internal granule layer (IGL) emerges to become the deepest cerebellar cortical layer at the end of organogenesis) (**A**) Clone H6-derived cells are present in the IGL ("**igl**"; **arrowheads**) of all lobes of the mature cerebellum in this parasagittal section. (Granule neurons are diminished throughout the cerebellum with some prominence in the anterior lobe). (**B**) Higher magnification of the representative posterior cerebellar lobe indicated by **arrowhead** "**b**" in (**A**), demonstrating the large number of donor-derived cells present within the recipient IGL. (**C-G**) Increasing magnifications of donor-derived cells (**brown nuclei**) within the IGL of a *mea* anterior cerebellar lobe. (Different animal from that in (A,B).) (**G**) Nomarski optics bring out the similarity in size and morphology of the few residual host, BrdU-negative cerebellar granule neurons (**arrowheads**) and a BrdU+, donor-derived neuron (**arrow**), which is representative of those seen in all engrafted lobes of all animals.) (**H, I**) Confirmation of the neuronal differentiation of a subpopulation of the donor-derived, BrdU+ clonal cells from (**A-G**) is illustrated by co-labeling with anti-BrdU (**green** in **H**) and the mature neuronal marker NeuN (**red** in **I**) (indicated with corresponding **arrows**). (Some adjacent, donor-derived cells are non-neuronal as indicated by their BrdU+ (**arrowhead** in (**H**)) but NeuN– phenotype. (**J**) Cells within the IGL are confirmed to be human donor-derived cells by FISH with a human-specific probe (**red**) identifying human chromosomal centromeres. Scale Bars: (**A**), (**B**): 100μm; (**F**), (**G**), (**J**): 10μm (Reproduced with permission from Ref. 28)

sure to serum), and proceeds gradually with neurons progressively increasing in number and extending processes over time (Figure 7D).

Taken together, these observations demonstrate that the human embryonic CNS contains neural progenitors that possess extensive self-renewal capacity and multipotentiality and that can legitimately, therefore, be classified as bona fide *human* NSCs. From a practical point of view, two complementary ways of establishing continuous lines of NSCs from the human CNS are now available to the research and clinical community. These human NSCs will likely represent a readily accessible, standardized, renewable source of human neural cells (particularly of functional human neurons) to be used for a variety of investigatory and therapeutic purposes.

Transplantation of Human Neural Stem Cells

Implantation in the neonatal rodent brain. A challenge to the neuro-repair community has been the replacement of brain cells lost to injury or disease (55). The attributes of NSCs would seem to make them well-suited to meet this challenge. Evidence of the capacity of NSCs to replace brain cells was initially provided in rodents by using rodent NSCs (110, 111, 113). With the isolation and cloning of human NSCs, the hope that this capacity is conserved at this higher phylogenetic stage was approached by our two groups by first implanting human NSCs in the brains of neonatal and adult rodents. Epigenetically- or genetically-expanded clones were deposited into the lateral ventricles of newborn mice. In striking similarity to their murine counterparts (110, 111), cells from the human NSC clones integrated into the SVZ and, from there, migrated extensively along the rostral migratory stream to the OB (a region of ongoing neuronogenesis) where donor-derived cells differentiated appropriately into interneurons (Figures 8, 9). Migration also occurred throughout subcortical and cortical regions (regions solely of gliogenesis at this developmental stage) where NSCs appropriately gave rise predominantly to astrocytes and oligodendrocytes (Figures 8, 9). These two alternative modes of differentiation adopted by the same clone highlights the plasticity of human NSCs and likely reflects their capacity to generate different cell types in response to the different cues that are present in various regions at specific stages of development . Similar results where observed when the identical clone of human NSCs was implanted at the opposite end of the neuraxis into the external germinal layer (EGL) of the cerebellum (Figures 10). Here cells mostly differentiated appropriately into an entirely different neuronal cell type, cerebellar granule cells of the internal granule layer (IGL). Using as a recipient the *meander tail (mea)* mouse mutant (which additionally provides a model of cell-autonomous degeneration and impaired development of granule neurons), it was possible also to demonstrate that human NSCs could, in fact, help reconstitute the neuron-deficient IGL.

Therefore, human NSCs, like their murine counterparts, seem to possess the capacity to contribute new neurons and glia seamlessly to the newborn brain, adopting a pattern of integration and differentiation that is developmentally-regulated and that mimics that of endogenous host cells (28). Furthermore, also like their rodent counterparts, human NSCs may be capable of neural cell replacement in conditions of neurodegeneration.

It is interesting and important to note that in the case of genetically-manipulated human NSC clones, the propagating gene product vmyc is undetectable in donor human cells beyond 24-48 hrs following engraftment (Figure 9Q-K) despite the fact that the brains of transplant recipients contain numerous stably-engrafted, healthy, well-differentiated, non-disruptive, donor-derived cells (Figures 8; 9A-J,Q; 10). This result was not unanticipated given that identical findings have been observed with v*myc*-propagated murine NSC clones (e.g. clone C17.2) in which v*myc* downregulation occurs constitutively and spontaneously and correlates with the typical quiescence of engrafted cells within 24-48 hrs post-transplantation. 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 spontaneously and constitutively 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 vivo* in mice (110, 114). With human NSCs, as with mouse NSCs, neoplasms are never seen.

Implantation in the adult rodent brain. As discussed earlier, similar to murine NSCs, human NSCs differentiate in a context-dependent manner by responding to local cues once engrafted in the host brain. With few exceptions (1, 10), neurogenesis is completed soon after fetal life throughout the mammalian brain. Hence, those cues that can drive the differentiation of human NSC-derived progeny into neurons are likely be extremely scarce within the CNS of adult graft recipients. To overcome this potential obstacle to achieving new donor-

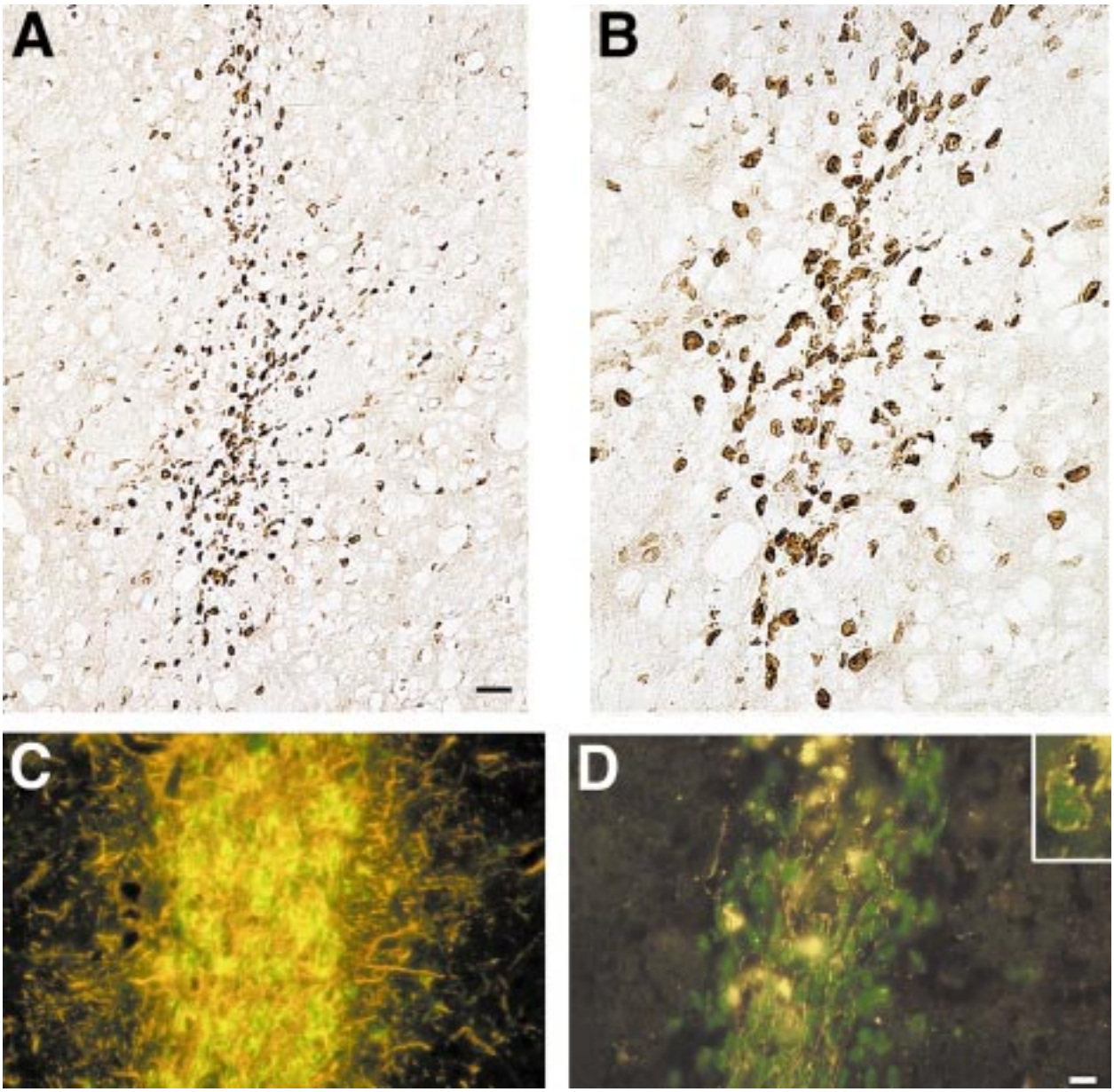

Figure 11. *Transplantation of human neural stem-like cells into the adult rodent CNS.* Human diencephalic stem cells (passage 32) were pre-labeled with BrdU and allowed to spontaneously differentiate *in vitro* prior to transplantation into the ipsilateral striatum of adult rats unilaterally lesioned with 6-OHDA (6.5 x 10^4 cells/graft) (128)). Grafted cells were detected by single immunofluorescence assays using an anti-BrdU antibody up to 4.5 months after grafting ((**A**); a high power magnification is shown in (**B**)). (**C**) Double immunofluorescence assays show that cells labeled with a human specific anti-ribonuclear protein antibody (AHN; **green**) co-express the astroglial antigen GFAP ((**C**); **orange**). Similarly, AHN-immunoreactive cells (**green**) co-express the neuronal antigen β-tubulin ((**D**), **orange**). (**D**) A higher magnification of one of the stem-cell derived human neurons is shown in the **inset**; the AHN antibody labels the nucleus with **green**, and the **orange** β-tubulin immunoreactivity is confined to the surrounding cytoplasm. Scale bars: (**A**), 20 μm; (**C**)-(**D**), 20 μm.

derived neurons in the adult rodent brain, human NSCs that were expanded epigenetically for over two years were "pre-differentiated" *prior* to their implantation (128). Cells were pre-labeled with BrdU while proliferating *in vitro* and were then differentiated in the absence of growth factors for 6 days. The resulting neurons and

glia were implanted into the ipsilateral striatum of adult rats lesioned unilaterally with 6-hydroxy-dopamine (6OHD). Surviving BrdU-immunoreactive cells could be detected for up to 1 year post-transplantation. Quantitative analysis of 5 animals sacrificed 4.5 months following transplantation revealed that ~10% of the injected human cells had survived (Figures 11A,B) (128) and that some had migrated as far as 1.2 mm rostro-caudally and 0.75 mm medio-laterally from the graft site. Importantly, the cells detected by the anti-BrdU antibody co-expressed human specific ribonuclear proteins or human mitochondrial markers proving that viable transplanted human cells could be effectively detected by antibodies raised against specific human antigens (128). Using the anti-human-ribonucleoprotein antibody in double-labeling immunocytochemistry experiments, the survival of transplanted human NSC-derived GFAP-expressing astrocytes and β-tubulin-expressing neurons could be affirmed, the former in greater abundance than the latter (Figures 6C,D) (128). In fact, the glial/neuronal ratio observed *in vitro* following pre-differentiation was reproduced *in vivo* following transplantation. (That no oligodendrocytes could be detected *in vivo* was likely due to the fact that this cell type represented a minimal fraction of the pre-differentiated cells originally transplanted.) Thus, human NSCs appear capable of contributing new neurons and glia to the adult CNS.

Our groups' observations on the plasticity of human neural progenitor cells has been supported by complementary findings by other investigators. Although not directly focused on the isolation of human NSCs, McKay and colleagues also observed incorporation by a polyclonal population of human neural progenitors into a variety of white and gray matter regions following intraventricular implantation into fetal rats (8) yielding donor-derived neurons, astrocytes, and oligodendrocytes and mimicking a pattern of engraftment shown for mixed populations of rodent cells. These observations taken together with those described above serve to reinforce the expectation that the functional attributes and therapeutic promise witnessed in rodent NSCs has been conserved by evolution in those cells of human origin.

Human Neural Stem Cells: Perspectives and Therapeutic Scenarios

Cell & gene replacement therapies. Transplantation of primary fetal neural tissue into the lesioned CNS is the most classical and direct approach to restoring lost nervous system function. Implantation of such neural cells has been directed to various animal models of such neurological disorders as Parkinson disease (80) and Huntington disease (85), spinal cord injuries (2), demyelinating disorders (22, 39) and cerebellar ataxia (141). One of the impediments to the progression of such neural transplantation from the experimental to the clinical stage has been the source of donor material (6). Surrounded by significant ethical issues, the procurement of requisite amounts of human fetal tissue is difficult. In addition, other variables such as age, storage, viability, contamination, and cellular composition can hardly be standardized, making the scheduling of elective surgery extremely difficult. To compound the issue further, material from multiple fetuses is usually required for a single transplant, thereby introducing even more heterogeneity to the donor grafts, increasing the probability of immunological rejection or contamination, and making the availability of sufficient graft material on demand much less likely (80). Finally, primary fetal tissue that does not produce sufficient amounts of a desired factor is exceedingly difficult to engineer genetically for the purposes of augmenting production of that molecule.

Stable clones of human NSCs can be safely and effectively propagated and expanded for at least three years. They can be reliably frozen, stored, transported, thawed, re-expanded to sufficient quantities as needed, and transplanted into multiple hosts on demand. (Their biosafety, sterility, and histocompatibility can be verified long before implantation which, in turn, can be electively scheduled.) When transplanted, they may differentiate into and replace damaged or degenerated neurons and/or glia. Further, host cells degenerating not only by cell-intrinsic disease processes but also cell-*extrinsic* forces might theoretically be replaced. For example, in some pathologic conditions, host factors may be elaborated within the microenvironment that would be inimical to the survival and/or differentiation of donor cells. However, donor NSCs may be engineered *ex vivo* to be resistant to these factors, or to secrete substances that might neutralize those factors, or to express trophic agents that might overcome those factors, perhaps allowing "sister" donor NSCs within the graft to differentiate into desired cells types. Thus, by providing a steady and renewable source of normal engraftable human precursors, neurons, astrocytes and oligodendrocytes, human NSCs appear to be an appealing alternative to fetal tissue as graft material.

The use of human NSCs provide other benefits to studies involving neural transplantation: they provide an unprecedented opportunity to control experimentally

some critical cell parameters (80). For example, cell differentiation can be initiated on the experimenter's own schedule and can be "fine-tuned" by modifying the culture medium. Thus, variables such as viability and cell composition can be monitored and manipulated and the characteristics of donor tissue "tailored" to fit specific applications. From a basic neuroscience point of view, investigators can readily genetically manipulate and

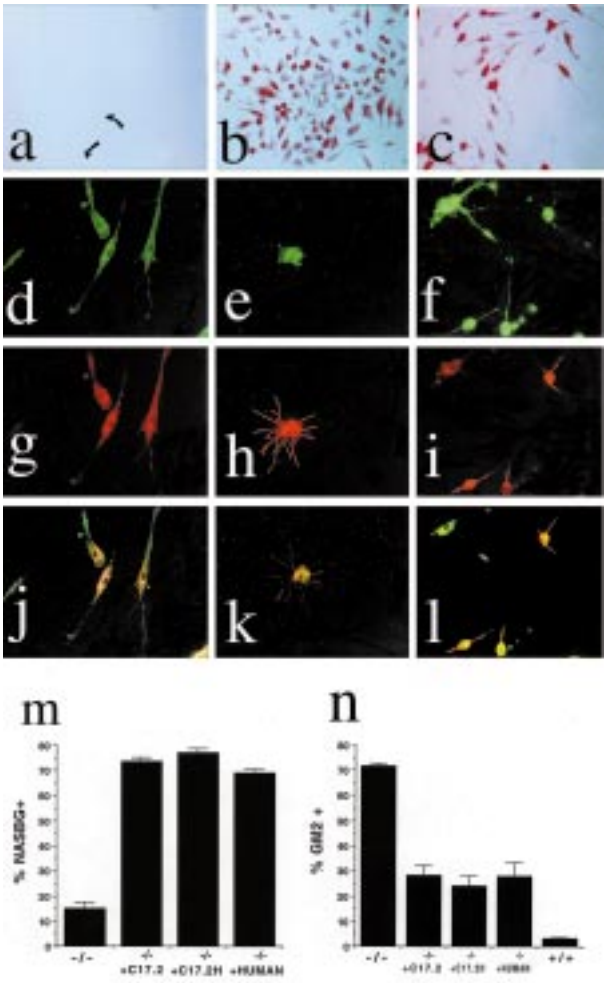

Figure 12. (Right) *Human NSCs are capable of complementing a prototypical gene product deficiency (e.g., β-hexosaminidase-A) in neural cells of multiple lineages in which the gene is mutated (e.g., brain cells from Tay-Sachs mice).* As a proof of principle that human NSCs (like murine NSCs) are capable of cross-correcting a neurogenetic defect, neural cells from the brains of mice with the prototypical neurogenetic disorder Tay-Sachs disease, generated via targeted mutagenesis of the α-subunit of β-hexosaminidase resulting in absence of hexosaminidase-A (138a), were exposed to secreted gene products from human NSCs to assess their ability to effect complementation of the defect. (**A-C**) Hexosaminidase activity as determined by NASBG histochemistry (Nomarski optics). Functional hexosaminidase produces a red-pink precipitate with an intensity proportional to the level of activity. (**A**) Tay-Sachs neural cells (**arrows**) not exposed to NSCs have no, or minimal, detectable hexosaminidase. (A small number of faintly pink NASBG+ cells are occasionally observed reflecting low residual hexosaminidase-B activity). In comparison, Tay-Sachs neural cells exposed to secretory products from murine NSCs (e.g., clone C17.2H) (**B**) or from human NSCs (**C**) now stain intensely red (wildtype intensity) suggesting that they have been cross-corrected, i.e., have internalized significant amounts of functionally active hexosaminidase from the NSC-conditioned medium. (**D-L**) To help determine which neural cell types from the Tay-Sachs brain were cross-corrected, primary dissociated Tay-Sachs neural cells which had been co-cultured in a transwell system with human NSCs (as in (**C**)) were reacted both with a fluorescein-labeled antibody to the human α-subunit of hexosaminidase (**D-F**) and with antibodies to neural cell type-specific antigens (visualized by a TR-tagged secondary antibody) (**G-I**, respectively). Photomicroscopy through a dual filter confirmed co-localization of the α-subunit with the cell-type markers (**J-L**, respectively). A subset of these now α-subunit-positive corrected cells (**D**) were neurons, as indicated by their expression of the neuronal marker NeuN (**G**, **J**); a subset of the α-subunit+ cells (**E**) were glial, as illustrated by their co-expression of the glial marker GFAP (**H**, **K**); and a subset of the α-subunit+ cells (**F**) were immature, undifferentiated CNS precursors, as indicated by the presence of the intermediate filament nestin (**I**, **L**). (Untreated cells from a Tay-Sachs brain do not stain for the α-subunit). (**M**) Percentage of successfully rescued (i.e., NASBG+) primary Tay-Sachs neural cells as seen in (**A-C**). The number of "untreated" Tay-Sachs α-subunit-null cells (-/-) (i.e., unexposed to NSCs) that were NASBG+ (**1st histogram**) was quite low. (That the percentage is not 0 reflects some low residual hexosaminidase-B activity in mutant cells that is sometimes sufficient enough in some cells to produce a pale pink scoreable cell). In contrast, among Tay-Sachs neural cells "treated" with secretory products from murine NSCs (C17.2) (**2nd histogram**), murine NSCs engineered to over-express hexosaminidase (C17.2H) (**3rd histogram**), or human NSCs (**4th histogram**), the percentage of cross-corrected, hexosaminidase-containing cells was significantly increased ($p<0.01$). The NSCs did not significantly differ from each other in their ability to effect this rescue. (NASBG staining of neural cells from a wildtype mouse served as a positive control and were nearly 100% NASBG+, histogram not presented). (**N**) Complementation of gene product deficiency results in rescue of a pathologic phenotype in mutated neural cells, as illustrated by percentage of Tay-Sachs CNS cells with diminished GM_2 accumulation. Among Tay-Sachs cells not exposed to NSCs (**1st histogram**), the percentage of GM_{2+} cells was large reflecting their pathologically high level of storage, corresponding to a lack of enzyme as per (**M**). In contrast, the percentage of cross-corrected Tay-Sachs cells without detectable GM2 storage following exposure to murine (**2nd** and **3rd histograms**, as in (**M**)) or human NSCs (**4th histogram**) was significantly lower than in the mutant ($p<0.01$), approaching that in wildtype (+/+) mouse brain (**5th histogram**). Again, the NSCs did not significantly differ from each other in their ability to effect this rescue. (Reproduced with permission from Ref. 28)

study molecules in these cells that may be pivotal for cell type specification by mutating specific genes in a targeted fashion, by transfecting the cells with dominant negative or positive forms of genes, or by blocking or overexpressing various ligands or receptors. Figure 7D illustrates the gradual maturation of stem cell-derived neurons; donor neurons can be collected and grafted at various stages in this developmental process to test the efficacy of their graft survival and integration. The spontaneous differentiation that occurs in these cultures can be manipulated (as in other systems (44, 88)) by exposure to various combinations of cytokines that might alter the ratio of neurons-to-glia (Figure 7C) (128) either in favor of neurons or in favor of particular types of glia for particular therapeutic demands (7, 71). Some CNS disorders may require grafting of specific neuronal subtypes. GABA'ergic neurons (the preferred neurotransmitter phenotype of NSC-derived neurons) might be useful for the replacement GABA-ergic spiny neurons in Huntington disease (85). The ability to promote a catecholaminergic phenotype in a significant fraction of the neuronal progeny of EGF-responsive rodent and human NSCs (18, 127, 128a) may prove useful for cell replacement therapy in Parkinson disease. Thus, in addition to reducing a dependence on primary human fetal brain tissue for transplantation, human NSC lines may also eliminate the need for obtaining cells exclusively from selected embryonic regions.

The fundamental biologic properties of NSCs, however, suggest that they may fulfill roles greater than simply being an alternative to fetal tissue in standard transplantation paradigms. For example, while neural transplantation has traditionally been reserved for diseases whose neuropathology reflects very anatomically restricted damage (e.g., the striatum in Parkinson disease), the use of NSCs, because of their ability to engraft within germinal zones and to migrate and integrate widely, may permit a cell-based therapy like neural transplantation to tackle CNS disorders that are extensive (like stroke or asphyxia), or multifocal (like multiple sclerosis) or even global (like most inherited metabolic disorders or dementing neurodegenerative diseases). Human NSCs, just as their murine counterparts, are capable of producing factors that can cross-correct genetic defects in brain cells from models of neurogenetic diseases (28) (Figure 12). Like their murine counterparts they can mediate the widespread expression of foreign transgenes following transplantation, migration to and stable integration and maturation within host parenchyma at distant sites. (Figure 9). Presumably, therefore, like their rodent counterparts whose success in many experimental models of neurological diseases has been affirmed, human NSCs, too, should represent optimal vehicles for disseminated CNS gene delivery — analogous to a "bone marrow transplantation to the brain". By virtue of their ability for widespread enzyme and/or cell replacement human NSCs should be able to address certain therapeutic challenges currently consigned solely to pharmacologic or genetic interventions. However, because NSCs appear also to incorporate into host cytoarchitecture in a functional manner, they should prove to be also more than vehicles for just the "passive" delivery of gene products: the regulated release of certain substances through feedback loops may be reconstituted, as might the reformation of essential circuits. Indeed, 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. Finally, because they can differentiate along multiple lineages and because many neurologic disorders are characterized by defects in multiple cells types, NSCs may help to reconstitute multiple aspects of a dysfunctional region.

It is interesting to recognize that even those implanted cells that do *not* differentiate into the desired cell types for a particular disease may serve a therapeutic function. Being of CNS origin, donor NSCs may intrinsically provide both diffusible and non-diffusible factors that might enable the injured host to regenerate its *own* lost cells and/or neural circuitry. When such factors are not naturally produced in sufficient quantities, the NSCs can be genetically engineered before transplantation to become "factories" for the sustained local production of substances known to mobilize quiescent host progenitor pools, promote the differentiation of immature nerve cells (either endogenous or, via paracrine/autocrine action, donor), attract ingrowth of host fibers, or forestall degeneration resulting from the insufficiency of a trophin or enzyme in the *milieu*.

Intriguingly, one multipotent NSC clone may, in certain conditions, theoretically perform all or many of the above-mentioned functions, even concurrently in the same transplant (e.g., (84)).

Conclusion

Studies over the past few years have suggested the existence in the mammalian CNS of neural cells with stem-like properties. The therapeutic applications of neural stem cells (NSCs) for disorders of the CNS may, indeed, be broad and multifaceted, analogous to the role played by the hematopoietic stem cell for systemic dis-

eases. To date, most insights into the NSC have derived from observations on rodent cells under the assumption that the attributes that endow these cells with their therapeutic potential are conserved in those of human origin. The recent isolation and propagation of stable clones of rigorously defined human NSCs has allowed investigators to confirm that such cells, in fact, appear to behave in a manner analogous to their lower mammalian counterparts suggesting that they, too, may have therapeutic potential. The similarity between human NSC clones and their murine counterparts in plasticity, genetic manipulability, responsiveness to regional and temporal developmental cues, migrational ability, foreign gene expression, cross-correctional ability, and neural cell replacement suggests that human cells may possess the range of capabilities to be as effective in true clinical situations as rodent NSCs are in animal models of many disorders. NSCs may also serve as adjuncts to other repair and gene therapy strategies using other cellular, viral, and non-viral vectors, or be used in conjunction with other more lineage-committed human-derived neural cells. Not only might the actual clones described in this review serve these various functions, but the data from our groups and others suggest that investigators may readily obtain and propagate such cells from their own sources of human material through a variety of equally safe and effective epigenetic and genetic methods with the expectation that they will fulfill the demands of multiple research and/or therapeutic problems by yielding engraftable, responsive neural cells for multiple hosts. Investigators can freely choose the technique that best serves their particular research or clinical needs. In helping to resolve debate over which tack is most effective for isolating and manipulating NSCs, the data reviewed in this review should also help unify various research directions: insights from studies of NSCs perpetuated by one technique may be legitimately joined to those derived from studies employing others, providing a more complete picture of NSC biology and its applications. With the development of human NSCs, we are becoming hopeful that progress towards human applications of the therapeutic paradigms discussed in this review may actually be in view.

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