Stem Cells, Regenerative Medicine, and Animal Models of Disease

Dennis A. Steindler

Abstract

The field of stem cell biology and regenerative medicine is rapidly moving toward translation to clinical practice, and in doing so has become even more dependent on animal donors and hosts for generating cellular reagents and assaying their potential therapeutic efficacy in models of human disease. Advances in cell culture technologies have revealed a remarkable plasticity of stem cells from embryonic and adult tissues, and transplantation models are now needed to test the ability of these cells to protect at-risk cells and replace cells lost to injury or disease. With such a mandate, issues related to acceptable sources and controversial (e.g., chimeric) models have challenged the field to provide justification of their potential efficacy before the passage of new restrictions that may curb anticipated breakthroughs. Progress from the use of both in vitro and in vivo regenerative medicine models already offers hope both for the facilitation of stem cell phenotyping in recursive gene expression profile models and for the use of stem cells as powerful new therapeutic reagents for cancer, stroke, Parkinson's, and other challenging human diseases that result in movement disorders. This article describes research in support of the following three objectives: (1) To discover the best stem or progenitor cell in vitro protocols for isolating, expanding, and priming these cells to facilitate their massive propagation into just the right type of neuronal precursor cell for protection or replacement protocols for brain injury or disease, including those that affect movement such as Parkinson's disease and stroke; (2) To discover biogenic factors—compounds that affect stem/progenitor cells (e.g., from high-throughput screening and other bioassay approaches)—that will encourage reactive cell genesis, survival, selected differentiation, and restoration of connectivity in central nervous system movement and other disorders; and (3) To establish the best animal models of human disease and injury, using both small and large animals, for testing new regenerative medicine therapeutics.

Key Words: drug discovery; human therapeutics; recursive gene profiling; regenerative medicine; stem cell; transplantation

Introduction

Stem cells have the propensity to produce tissue, an attribute that not only contributes to normal human development but also can lead to oncogenic transformation and hyperplasia (Gibbs et al. 2005; Ignatova et al. 2002; Steindler 2006). Two characteristics of stem (and progenitor) cells reveal their dual nature: (1) "poiesis" (generation) and (2) the overgeneration of cells and tissue (the so-called oncogenic transformation that leads to neoplasia). Because of these particular attributes, there is widespread interest in stem cells and regenerative medicine and their potential to treat and cure human diseases. But controversy and debates surround the question of which cells might be both the best and the most ethically acceptable therapeutic reagents, likewise determining which animal models are indeed the most effective. Animal models of disease are certainly necessary for the regenerative medicine field. Clinical trials of adult (e.g., bone marrow or cord blood transplantation) as well as fetal stem or progenitor cells have already demonstrated the efficacy of such regenerative medicine cell therapies for protecting, repairing, and replacing at-risk cells and tissues (Bjorklund 2005; Reier 2004; Press Release, Yahoo! Finance, November 15, 2006). Yet there is also a daunting side to the emerging field of regenerative medicine. Great expectations and desperate hope for immediate clinical application have driven intense debates at the state level, and international hearings to establish guidelines also try to respond to demands from different citizen groups with disparate agendas. The situation has also prompted patients all over the world to seek alternative and usually unproven stem cell "therapies" that can put them at risk. These challenges justify support for more science that must include both in vitro and in vivo studies of stem/progenitor cells from a variety of tissues and organs.

This article describes advances to date in the use of cells and animal models in regenerative medicine, expectations for future discoveries of the best stem and progenitor cell populations from different tissues and organs, and how in

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vitro high-throughput screening (HTS[1]) bioassays might best utilize the potency of embryonic, fetal, and adult stem cells. The article also describes uses of modeling, in vitro studies, and dynamic stem cell and biogenic stem cell factor screening that could lead to more rapid developments in translational regenerative medicine. The reasoning below suggests that research in regenerative biology and regenerative medicine, although human-centric because of the eventual need for cells from a variety of human tissues and organs at different stages of development and aging, nonetheless requires animals, both as sources of immature cells and as recipients for cell and engineered tissue grafts to establish therapeutic proof-of-principle for any new cell or drug therapy. In vitro bioassay screening and use of simpler organisms could reduce the need for experimentation with mammalian models once researchers better understand the nature of different stem/progenitor cell populations and also further refine HTS. Thus, it is worthwhile to further develop cell culture assays, explore virtual gene and protein screens, and establish standardized and efficient rodent and other animal models of human disease to generate universal bioassays that can be used to establish the required safety and efficacy of any potential new regenerative medicine therapy before going on to human clinical trials. In particular, immunocompromised animals with diseased and injured tissues should continue to host human cell transplants, and investigators should continue to test new drugs gleaned from studies of the bioactive compounds associated with the growth and differentiation of stem cells in the same animal models.

There is no question that animal models of stem cell research in support of regenerative medicine will facilitate rapid translation to the bedside. The regenerative medicine field will continue to foster respect for the animal kingdom amid a pressing need to find new cures for human suffering. With the remarkable paradigm shift that has occurred in scientists' understanding of human self-regenerative potential, there is a high level of confidence that stem cell biology and regenerative medicine will lead to exceptionally effective new therapeutics for movement disorders and all other neurological challenges in the not too distant future.

In Vitro Studies of Stem and Progenitor Cells

The notion of stem cells having clinical relevance is certainly not new. Since the 1950s investigators have performed adult stem cell transplants with impressive curative potential for cancers and a variety of other blood-related diseases. However, the concept of global human regenerative potential and the birth of the regenerative medicine field are fairly recent. With profound discoveries and advances in genetics and molecular biology, the field of developmental biology has benefited tremendously. Among the animal models that have long played an important role in biology are both invertebrates (e.g., *Drosophila*, *Aplysia*) and vertebrates (e.g., Zebrafish, rodents), as well as mutant and transgenic animals that have revolutionized scientists' ability to characterize normal, abnormal, and restorative development. Research on bone marrow hematopoiesis began in the 1950s (e.g., Metcalf 1959), with the pioneering discoveries of radiation effects on a cell population that has blood and tissue regenerative capacities (for review, see Steindler 2006). The early studies of stem cells in dishes from hematopoietic bone marrow established cell culture protocols that are still in use (e.g., the work of Dexter, Metcalf, and others, discussed in Scheffler et al. 1999). This research also provided insights and templates for extending the notion of a population of primitive cells that gives rise to other cells (hence the name "stem") and to all tissues, not only blood. A review of the nature of stem and progenitor cells from all tissues is beyond the scope of this article; rather, the focus here is "neuropoiesis," or the lifelong neurogenic potential of cells in embryos, fetuses, and adults that contribute to the formation of the central nervous system (CNS[1]) and also seem poised to replace lost CNS circuitry elements after injury or neurodegenerative disease. With a lexicon born in the field of developmental biology and the need to understand how cells and molecules interact to generate tissue, it is feasible to view the field of regenerative medicine as translational developmental biology that now requires novel in vitro and in vivo bioassays to bring the work from the bench to the bedside.

Stem cell biology has its own lexicon, often confused by uncertainty in establishing degree of "stemness" (i.e., whether a cell truly meets the stringent definition of a stem cell or rather exhibits some but not all stem cell behaviors) and by sometimes ambiguous phenotype of the progeny of stem or progenitor cells. Numerous reviews on stem cell biology have addressed the issue of a continuum of cell fate or differentiation from the most primitive precursor cell to the most differentiated adult somatic cell (e.g., neurons and glia) (see Scheffler et al. 1999; Steindler 2006; and Gage 2000 for a complete list of definitions of stem cells and their progeny). Rather than defining the differences between a stem cell, a progenitor cell, and a terminally differentiated cell, this article focuses on the stem cell as the most potent of all cells. It has the ability to self-renew, give rise to more committed progeny ("progenitors"), respond to tissue injury or disease with reparative attempts, and maintain all of these characteristics even after serial transplantation. Although a progenitor cell is more committed, the word also applies to stem cells (i.e., stem/progenitor cells) when the degree of "stemness" is not certain. An emerging concept is that ap-

[1]Abbreviations used in this article: AHNP, adult human brain neural progenitor; CNS, central nervous system; ES, embryonic stem; ESNPs, ES cell-derived neural precursors; FDA, US Food and Drug Administration; hESCs, human embryonic stem cells; HIE, hypoxic-ischemic encephalopathy; HTS, high-throughput screening; MASC, multipotent astrocytic stem cell; PCR, polymerase chain reaction; SCNT, somatic cell nuclear transfer; SSEA, stage-specific embryonic antigen.

parently fully differentiated cells are recognizable by their expression of a variety of tissue/cell-"specific" markers and their ability to dedifferentiate or transdifferentiate to a variety of states and midpoints (e.g., Laywell et al. 2005). It is therefore now even more difficult to assign both stemness and phenotypy in a completely reliable way (Steindler 2006), and thus words like "precursor" emerge as a way to describe cells that are in transition between being proliferative and seeking fate and commitment.

Most of what the research community knows about the nature and behaviors of stem/progenitor cells comes from in vitro studies of these cells, subjecting them to growth factor and other morphogenetic molecule concoctions that attempt to mimic as much as possible growth conditions in vivo. But many of the events that investigators observe and attempt to characterize in a dish are not as they occur in life because the cells are exposed to extremely nonphysiological amounts of potent growth factors, cytokines, and morphogens that have profound effects on their choices to divide or differentiate along a particular line. Even so, the recent in vitro studies in which we and others have been involved achieve a rather impressive level of recapitulating many, and in some cases (Scheffler et al. 2005) all, of the cell-cell interactions and differentiation cascades that lead to the generation of normal cell lineage diversity for a particular (e.g., forebrain) circuit as it evolves in vivo. Numerous studies have suggested the possibility of using different stem/progenitor cell populations as either factor delivery systems or cell replacements for different human diseases.

My laboratory has recently exploited novel cell culturing along with immunophenotyping and functional characterization (patch clamp recordings) to show a transition from embryonic to neuronal and glial cell differentiation that is profoundly affected by environmental cues (e.g., morphogenetic extracellular matrix molecules) to help guide fate choice including forebrain GABAergic phenotype, migration, and neurite extension of neural precursor cells derived from embryonic stem (ES[1]) cells (Goetz et al. 2006). It is clear that ES cell biology holds tremendous promise for revolutionizing cell and molecular medicine. Our recent paper (Goetz et al. 2006) also exemplifies the tremendous potential for understanding ES cell biology and potential from the use of both animal (in this case, mouse) and human sources. It seems early in the evolution of this field to confine the research to one species source or a single cell line when trying to establish the reparative efficacy of ES cell-derived cells for tissue repair.

The ability to isolate, enrich, and expand stem/progenitor populations from the postnatal and adult mammalian CNS is also particularly important for studies attempting to characterize and determine efficacy of these more "mature" cells in protection and replacement protocols. The study of these cells has focused on the periventricular subventricular zone (SVZ[1]) of the rostral mammalian forebrain. The adult SVZ seems to be a special region because it represents the vestigial embryonic germinal zone, displays a high level of constitutive proliferation, and is likely to contain the greatest density of putative neural stem cells. Work in my laboratory (Laywell et al. 2000) has shown that a cell exhibiting characteristics of an astrocyte, from the entire developing brain until the end of the second postnatal week, and in the SVZ throughout life, is a multipotent stem cell (or multipotent astrocytic stem cell, MASC[1]) that can give rise to neurospheres containing both glia and neurons. A MASC is also a good candidate for cell replacement transplantation because it represents the indigenous neural stem cell that is amenable to ex vivo expansion and potentially even autologous grafting approaches (see Transplantation of Neural Stem/Progenitor Cells, below).

In my laboratory we have also recently demonstrated a new method to isolate and evaluate phenotypic and functional features of adult brain neurogenesis in vitro (Scheffler et al. 2005; Figure 1). These "founder" cells of persistent forebrain neurogenesis can be isolated from the mouse SVZ and expanded in plastic culture dishes using serum-containing media and mitogenic factors (EGF+FGF-2). Upon withdrawal of the serum and mitogens, the cells undergo rapid glial-to-neuronal phenotype transition and yield type-A precursor cells (Doetsch et al. 1999), or "neuroblasts," specific to the SVZ that mature to a more neuronal phenotype with time in culture. This in vitro protocol can generate unusually large numbers of neuroblasts that exhibit many if not all of the characteristics of forebrain neuronal precursor cells as seen in vivo. The resulting cells can be used in bioassays to screen for compounds that encourage neuropoiesis and directed differentiation of the cells into particular neuronal phenotypes as well as for transplantation in animal models of neurological disease. It is characteristic for the adult SVZ in vivo to generate precursors that mature to GABAergic neuronal phenotypes, but it was surprising that the same fate restriction appeared to be present in a dispersed cell culture setting (Scheffler et al. 2005).

Our most recent studies (Walton et al. 2006) support the notion of possible dedifferentiation in mature, differentiated populations of neural cells (e.g., a subclass of the pervasive astrocyte population). Our research also suggests cell culture methods that facilitate enrichment of a very small number of stem/progenitor cells that inhabit the gray matter of the cerebral cortex of adult humans when placed under particular growth conditions that maintain telomerase expression without immortalization. Furthermore, our findings support recent observations of a potential lack of replicative senescence (the possible challenge of the "Hayflick Limit"), which suggests that cells can undergo only so many population doublings (e.g., 50 divisions) before the senescence of these clonogenic, multipotent cells (Walton et al. 2006). Despite many holes in our understanding of the nature of adult neural stem cells, they do offer a potential source of cells for drug-screening bioassays as well as for transplantation in models of neurological disease. Our finding that both adult mouse and human neural stem/progenitor cells can survive in tissue with rather protracted postmortem intervals (Laywell et al. 1999) even suggests that the cadaveric human brain is a source of cells for possible ma-

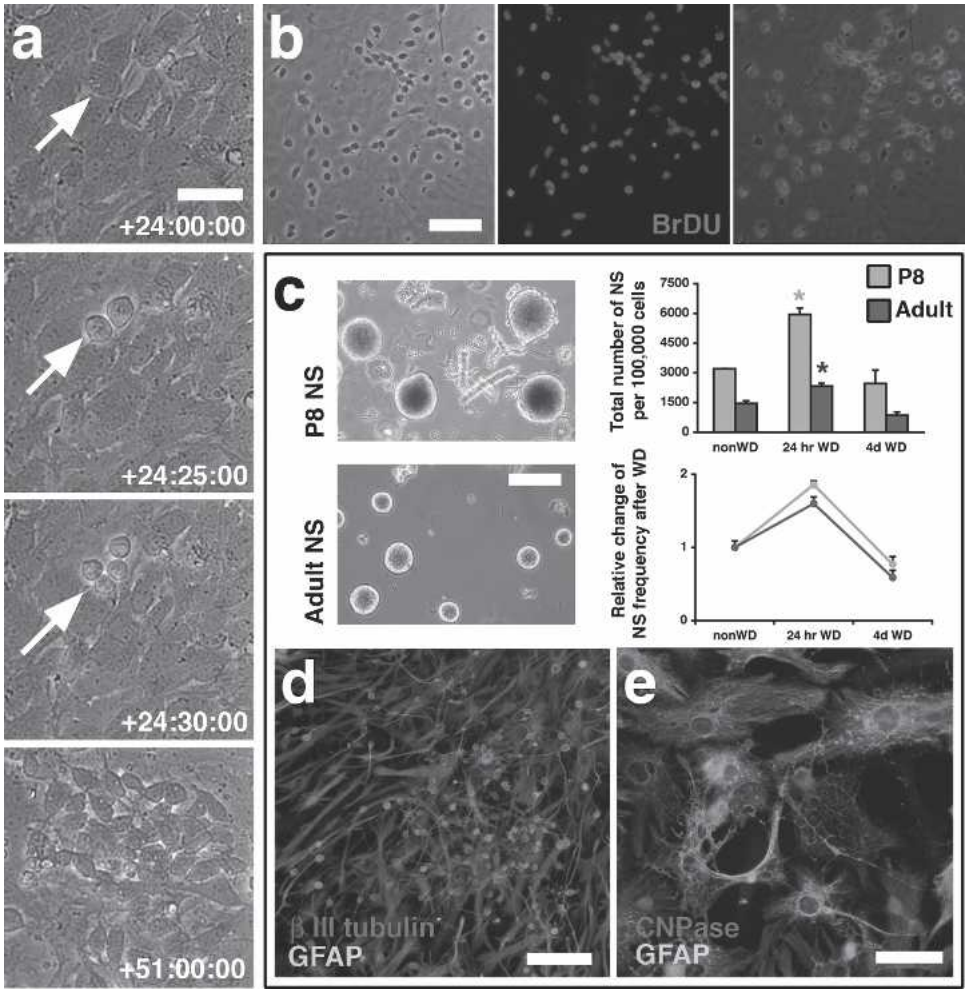


Figure 1 Subventricular zone (SVZ) neurogenesis in vitro. This is an inducible neurogenesis bioassay, also called a "neuroblast assay" because large numbers of neuroblasts can be generated in this culture system. Mitotically active multipotent cells emerge transiently during early stages of the in vitro assay that accurately mimic SVZ neuropoiesis as it occurs in vivo. (A) Time-lapse microscopy of growth-factor withdrawn SVZ cultures reveals a transient period, characterized by rapid cell divisions, leading to the initial appearance of neuroblasts within 27 hours. (B) The thymidine analog BrdU that labels dividing cells, applied at 48-72 hours after the initiation of differentiation, labels 95% of all generated neuroblasts. (C) Clonal neurospheres (NS) derived from cultured postnatal day 8 (P8) and adult SVZ cells (left, for morphological comparison). Total numbers and relative frequencies (right) of neurospheres generated from 100,000 cells per condition increase significantly but transiently at 24 hours after growth-factor withdrawal (WD) (*, $P < 0.01$ for adult and P8 compared with non-WD and 4-d WD). (D and E) Primary and secondary P8 and adult (shown here) neurospheres yield neurons (ß-III tubulin+ in D) and glia (GFAP+ astrocytes in D and E, CNPase+ oligodendrocytes in E). [Scale bars (in μm): (A), 15; (B), 60; (C), 200; (D and E), 20.] Reproduced with permission from Scheffler B, Walton NM, Lin DD, Goetz AK, Enikolopov G, Roper SN, Steindler DA. 2005. Phenotypic and functional characterization of adult brain neuropoiesis. Proc Natl Acad Sci U S A 102:9353-9358. Epub 2005 Jun 16. PMID: 15961540 [PubMed—indexed for MEDLINE]. The original figure is in color and the reader is directed to Scheffler et al. *(*2005) for details.

nipulation in therapeutic applications for many diseases. Adult human brain neural progenitor cells (Walton et al. 2006) that exhibit a significant amount of astrotypy and that in our pilot studies give rise to very large numbers (Figures 2 and 3) of normal, nontransformed cells for cell or combined cell/gene therapy are extremely well-suited for studies of CNS therapeutics (Steindler 2006; Walton et al. 2006). These cells can undergo expansion to a large scale without immortalization, and we also have a great deal of pilot data on their molecular and physiological phenotype (Walton et al. 2006; Figures 2 and 3).

State-of-the-art high-throughput small molecule screening (HTS) can begin following establishment of the best in vitro culture system, with the most appropriate stem/progenitor or precursor cell of interest. The in vitro methodologies described above, which maximize cell expansion and controlled differentiation protocols, enable the most efficient implementation of animal and human tissue donors possible for HTS and other screening approaches. In addition, various robotic approaches (e.g., Kalypsys automated small molecule screening systems) are capable of screening hundreds of thousands if not millions of compounds per

day. Commercially available drug libraries offer natural compounds as well as US Food and Drug Administration (FDA[1])-approved drugs for application in HTS bioassays. Recent successes with drug screens (Rothstein et al. 2005) offer fair degrees of confidence that bioassays such as the neuroblast assay provide a clear-cut readout that can yield effective drugs and factors for further testing in organ cultures and in vivo animal testing. In addition, the application of fluorescence-activated cell sorting and automated readouts to HTS for novel cell and molecular therapeutic discovery affords rapid and efficient analyses of data generated from precursor cells derived from potentially any tissue source. A recent study successfully applied this approach and discovered a new small molecule that facilitates propagation of ES cells (Chen et al. 2006) that might help in the expansion of human ES cells without feeder layers. Another recent study (Takahashi and Yamanaka 2006) took four genes from profiling and screening studies and inserted them into a somatic cell to generate ES-like cells without any application of somatic cell nuclear transfer (SCNT[1]), resulting in a possible new method to generate important ES lines without blastocyst generation. Such information on factors gleaned from HTS or other screens that might affect stemness, stem cell proliferation, and neurogenesis, along with gene discovery approaches described below, can enhance the generation of neural and other precursor cells as well as prime them for controlled differentiation into different populations of neurons and glia that are potentially amenable to transplant therapies for particular neurological disorders.

Recursive Gene Discovery on Stem and Progenitor Cell cDNA Panels

One goal of the field of stem cell biology and regenerative medicine is to apply genomics, proteomics, transcriptomics, and metabolomics to precursor cells in order to understand how they multiply, grow, differentiate, and survive. In doing so, we will discover not only cell and molecular interactions important or necessary for understanding the developmental biology of cells and tissues but also "biogenic" factors involved in controlling these important cellular processes and histo- and organogenesis. After all, the goal of research in regenerative medicine is to make it possible to rebuild human tissues and organs that have succumbed to injury or disease. One could hypothesize that if an investigator had a source of gene expression profiling reagents that could be continuously interrogated for gene expression patterns of interest, there would be less reliance on continually generating animal or human cell/tissue live reagents for molecular phenotyping studies of stem/progenitor cells. Since the pioneering studies of Lemischka's group on gene expression of stem cells (Ivanova et al. 2002) and more recently on neural stem cells (Shen et al. 2006), a goal of neuropoiesis research has been to establish a dependable procedure for the discovery of genes expressed during neurogenesis. Any insights into their temporal expression—e.g., revealing morphogenetic genes that turn on and off during critical times in stem/progenitor cell development—would also be tremendously helpful for attempting to control the development (priming) of these cells for transplantation therapies.

We have previously described (Suslov et al. 2000, 2002) the manipulation of cDNA libraries to generate dynamic gene expression panels from a heterogeneous population of neurosphere-initiating cells. The panels of cDNA libraries come from multiple neurospheres, clones of cells derived from a single stem/progenitor cell grown under particular in vitro conditions that favor the survival and propagation of only such a clonogenic cell (Kukekov et al. 1999; Reynolds and Weiss 1992). The neurospheres, at different stages of growth and differentiation, seem to contain transcripts of all genes involved in stem/progenitor cell division, expansion, growth, differentiation, and survival/death. This variety should make possible the discovery of temporally regulated gene expression in vitro that could recapitulate neuromorphogenetic gene expression as found in vivo.

Current research focuses on the pluripotency and self-renewal capabilities of neuropoietic cells, despite the relative paucity of markers that would enable the categorization of these cells in a manner similar to that used for hematopoietic stem/progenitor cells. Subsets of neurospheres may express distinct markers, inasmuch as it is possible to immunolabel stem cells from hematopoietic and other germinal sources with the different carbohydrate-recognizing stage-specific embryonic antigen (SSEA[1]) antibodies (Shamblott et al. 1998; Thomson et al. 1998). Immunophenotypic analysis of cultured embryoid bodies reveals a "programmed sequence of cell surface marker display" associated with the development of embryonic cell lineages (Ling and Neben 1997). A similar pattern of distinct molecular expressions seems to accompany neurosphere growth and maturation in vitro.

Neurospheres represent distinct clonal units that arise from stem/progenitor cells in particular stages of maturation. Every neurosphere represents the clonal expansion of cells that originated during distinct ontological stages of neural development. Heterogeneous populations of neurospheres (Kukekov et al. 1999; Suslov et al. 2002) should comprise mixtures of cells in miscellaneous stages of differentiation. Markers of hemato- and neuropoietic cells themselves offer valuable insights into the molecular bases of stem/progenitor cell fate and growth. Neurospheres represent a reliable model for gene discovery studies (Suslov et al. 2002), as it is possible to isolate the markers and genes expressed by even some of the most immature hematopoietic (e.g., CD34, stem cell factor) and neuropoietic (certain cytoskeletal proteins, e.g., nestin, McKay 1997; tenascin and *Pax-6,* Kukekov et al. 1999) stem/progenitor cells for use in subsequent gene and molecular analyses. For example, using the cell surface marker PSA-NCAM, Rao and collaborators (Mayer-Proschel et al. 1997) have used a panning method to isolate neuronal-restricted precursor cells.

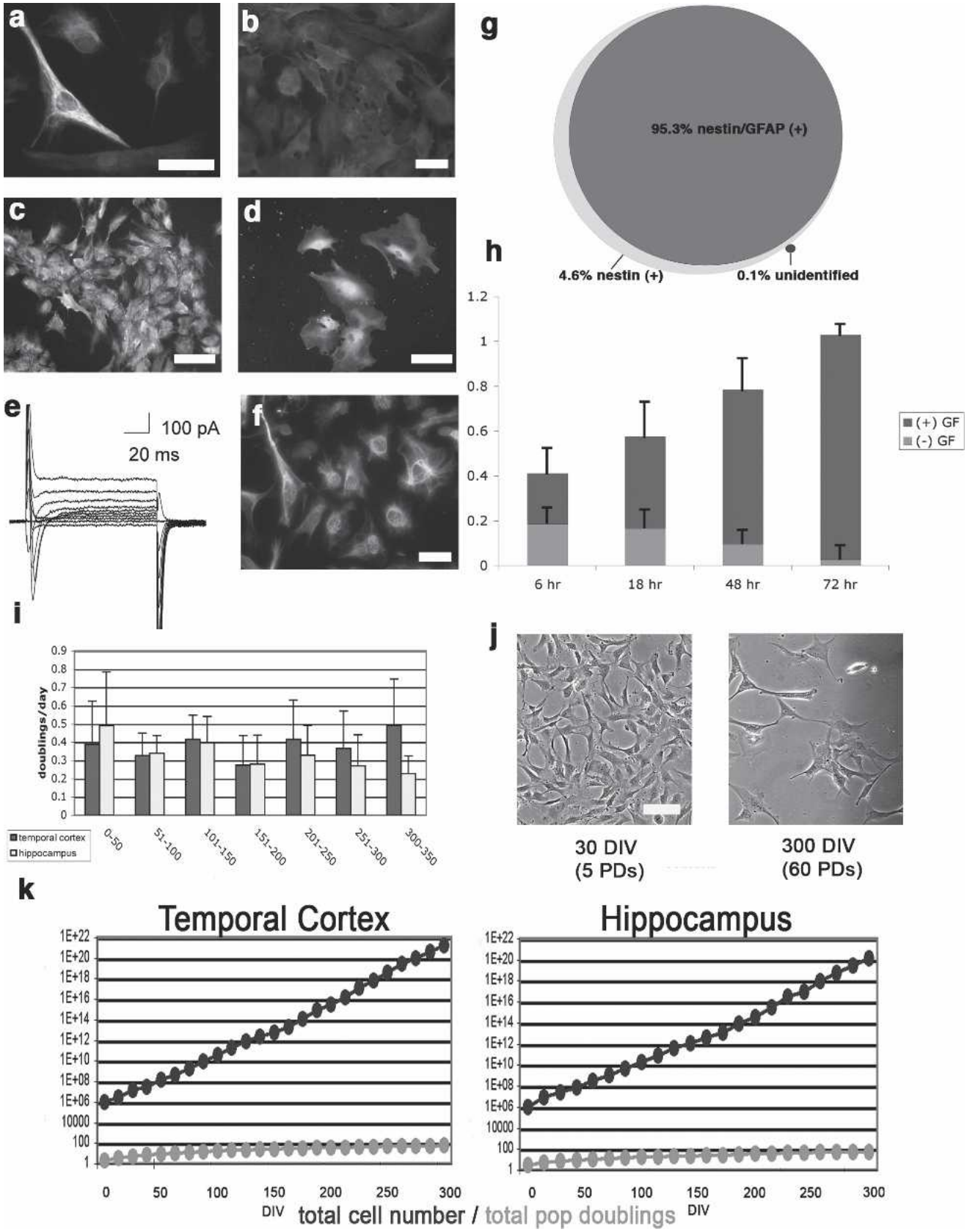


Figure 2

This and similar approaches enable the characterization of existing or novel genes involved in the commitment and maturation of particular populations of CNS cells.

Thus panels of cDNA libraries from a spectrum of differentiating neurospheres contain a full set of transcripts of genes responsible for cell proliferation and fate decisions as seen during in vivo neuromorphogenesis (Figure 4), and microarray screening and reverse transcriptase (RT)-polymerase chain reaction (PCR[1]) studies enable the continuous addition of new transcripts. Moreover, the ability to generate neurospheres from autopsy specimens with extended postmortem intervals (e.g., up to 5 days; Laywell et al. 1999) provides the opportunity to create panels of cDNA libraries from neurologically abnormal stem/progenitor cells (e.g., neurodegenerative diseases such as Alzheimer's and Huntington's). We can also use ES cells to generate such panels, as our recent study has demonstrated the use of RT-PCR to discover genes that provide brain positional fate to ES-derived neural precursors (Goetz et al. 2006). In addition, the application of such information to cDNA panels from these cells generates a dynamic gene expression profiling system that is applicable to any developing cell system.

This approach enables follow-up studies that incorporate similar methods to confirm distinct gene expressions across clones (and potentially single cells), focusing on novel transcripts for later sequencing in order to confirm gene discovery. Differential and subtractive (e.g., SSH; Suslov et al. 2002) methods to identify gene expression that varies across neurospheres or individual stem/progenitor cells should reflect temporal variation in gene expression (including the turning on and off of particular genes). Thus the generation of cDNA libraries from individual stem/progenitor cell clones that are part of a continuum of developmental states offers a potentially powerful approach for recursive probing of gene expression patterns of identified stem/progenitor cell populations. This of course does not represent an alternative to the use of animal or human tissues for molecular fingerprinting studies, but it does represent an approach that can reduce the numbers of animals or specimens required to perform multiple, iterative interrogations of gene transcripts present in a given population of potent cells at the precise moment of RNA extraction, and it provides insights into the sequence of events that lead to activation of particular genes (Suslov et al. 2002).

Transplantation of Neural Stem/Progenitor Cells

After the refinement of the in vitro studies of different stem/progenitor cell populations to generate cells that are as homogeneous as possible as well as highly expandable and potentially fate-controlled, and once the phenotyping and gene/factor screening experiments have yielded molecular candidates for enhancing cell genesis and precursor (e.g., neural) priming, it is feasible to use the results of in vitro studies for in vivo models, for both transplantation and drug testing paradigms. The choice of which cell is best for the protection or replacement protocol is crucial, in keeping with previous clinical trials. For example, for Parkinson's disease (see Bjorklund 2005 for review) a cell that in animal model studies seemed like a natural for therapeutic replacement of dopamine in the human condition (the fetal midbrain dopamine neuron precursor) may not live up to expectations. There have been cases of both remarkable improvement after fetal midbrain transplants for Parkinson's disease and debilitating dyskinesias following the transplantation protocol. Such variable results indicate the need for more research to determine how to maximize cell therapy for this and other neurodegenerative diseases. Many investigators and studies have suggested that the best approach for treating a movement disorder such as Parkinson's would be to rescue and protect at-risk dopamine neurons, and then eventually attempt reconstitution of the dopaminergic nigrostriatal axis.

Complexities and problems associated with the human fetal mesencephalic transplant trials—including variation among protocols at different institutions (e.g., in preparation of the grafts, additions of certain growth/survival factors) and differing viewpoints for graft placement—are all possible reasons for different outcomes. The clinical trials in Parkinson's disease that used fetal tissue achieved a degree

Figure 2 Expansion of primary neural "astrotypic" cells as a homogenous population of adult human brain neural progenitor cells (AHNPs). (A) Highly expanded (more than 60 passages, PDs) cells ubiquitously express nestin, with a large subset of GFAP+ cells. (B) AHNPs express widespread immature neuronal and glial markers, including A2B5 and NG2. (C,D) AHNPs (nestin+) express astrotypic markers in a large subset of cells, including S100 (D). (E) Voltage-clamp profile of these cells reveals prominent Na+ and K+ channel activity. Data shown for temporal cortex-derived cells. (F) Nestin+ AHNPs proliferated in the presence of BrdU uniformly incorporate this thymidine analog. (G) Stereological evaluation of proliferating AHNPs reveals a uniform nestin+ population that frequently coexpresses glial cell markers (GFAP shown). Maintaining these cells in growth medium supplemented with BrdU results in label saturation in AHNPs (BrdU+/Nestin+ cells) at a rate of incorporation analagous to previously characterized proliferative dynamics (H). Removal of mitogenic stimuli (GF = EGF+bFGF) results in failure of AHNPs to divide (also see F). (I,J) Both hippocampal and temporal cortex-derived AHNPs maintain comparable stable doubling rates and uniform protoplasmic morphologies throughout culture. (K) AHNPs derived from temporal cortex and hippocampus reveal continuous logarithmic expansion throughout culture. Scale bars: 25 μm (A-D, F), 75 μm (J), DAPI-counterstaining. Reproduced with permission from Walton NM, Sutter BM, Chen HX, Chang LJ, Roper SN, Scheffler B, Steindler DA. 2006. Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain. Development 133:3671-3681. Epub 2006 Aug 16. PMID: 16914491 [PubMed—indexed for MEDLINE]. The original figure is in color and the reader is directed to Walton et al. (2006) for details.

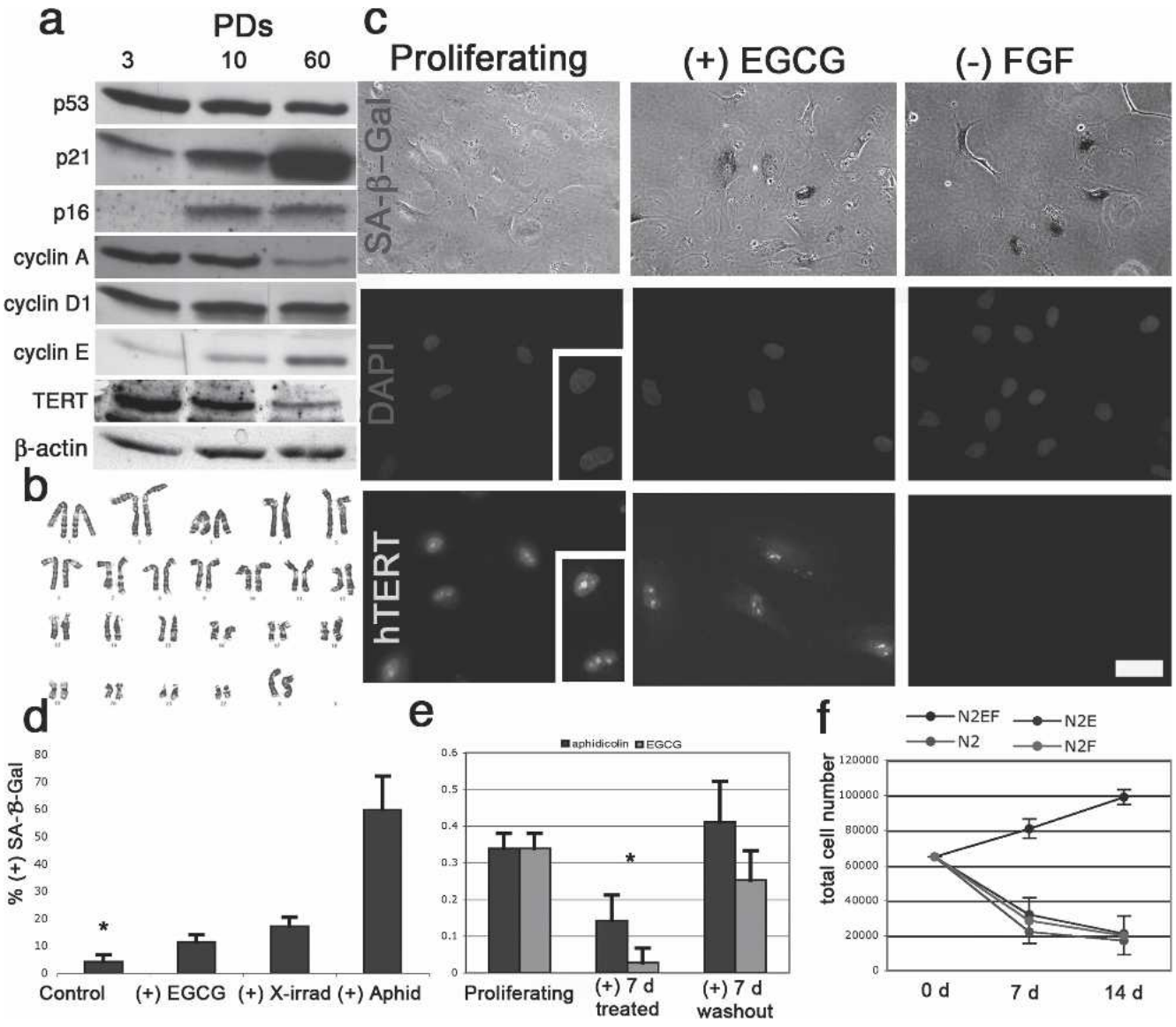


Figure 3 AHNPs avoid immortalizing mutations, and exhibit mitogen- and telomerase-dependent growth. (A) Cultured AHNPs express major growth regulatory proteins longitudinally throughout culture. (B) Karyotyped AHNPs display normal ploidy and have no gross cytogenetic malformations. (C) Following growth arrest by an exogenous TERT inhibitor (EGCG) or growth factor withdrawal, cultured cells express SA-ß-gal. However, only mitogen-withdrawn (–bFGF) cells lose TERT expression when evaluated 7 days later. (D) Physiological (x-irradiation) or chemical inhibitors (aphidicolin, EGCG) consistently increase the fraction of cells expressing SA-ß-gal (E) Application of reversible growth inhibitors yields a significant reduction in growth rate. AHNPs revert to previous proliferative levels following arrestor washout. (F) Age-matched AHNPs placed in either basic media (N2) or media containing EGF or bFGF only (N2E, N2F) enter irreversible growth arrest compared with defined proliferative conditions (N2EF) and subsequently become unviable. Data shown for temporal cortex derived cells. *$P < 0.05$, Student's t-test. Scale bar: 75 μm. Reproduced with permission from Walton NM, Sutter BM, Chen HX, Chang LJ, Roper SN, Scheffler B, Steindler DA. 2006. Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain. Development 133:3671-3681. Epub 2006 Aug 16. PMID: 16914491 [PubMed—indexed for MEDLINE]. The original figure is in color and the reader is directed to Walton et al. (2006) for details.

of success, and the transplanted tissue contained neural stem and progenitor cells. Animal model studies using grafts of fetal mesencephalon included immature neurons, precursor cells, and even perhaps stem cells. Reier and colleagues made use of this knowledge when they participated in the first human fetal cell transplants for spinal cord injury (see Reier 2004 for review). The procedure explicitly recognized the advantage of the presence of stem/progenitor cells, and the outcomes of this trial were positive with regard to both safety and potential efficacy. The presumption is that stem cells in these tissue grafts will either eventually differentiate into neurons and glia or die as a result of the mature brain's

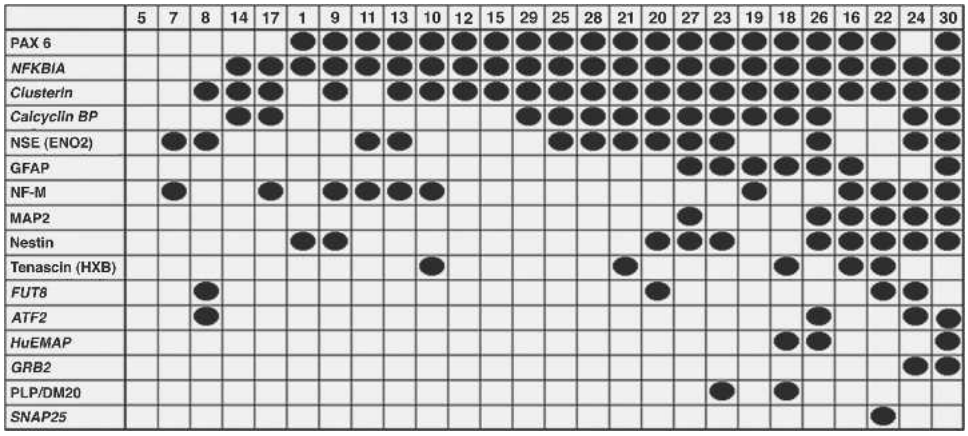


Figure 4 A cDNA panel developed for dynamic and recursive gene screening with transcripts expressed by human neurosphere cells. Genes can be continually added (left side of the panel) as they are discovered using microarray screening and PCR, and the panel interrogated following hybridization with new cDNAs. There is clear heterogeneity of these 30 different neurosphere clones derived from 30 stem/progenitor cells isolated from the same adult human brain dissociation. Some clones (e.g., #30) seem to express primitive markers of stem cells (e.g., nestin, Pax 6, tenascin) that make this clone more "immature." Cross-comparing clones and performing subtractive and differential expression affords temporal profiling almost ad infinitum. Reproduced with permission from Suslov ON, Kukekov VG, Ignatova TN, Steindler DA. 2002. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. Proc Natl Acad Sci U S A 99:14506-14511. Epub 2002 Oct 15. PMID: 12381788 [PubMed—indexed for MEDLINE].

altered molecular environments, which are not conducive for stemness.

More such studies, as well as long-term patient follow-ups, could help to corroborate and provide additional insights into graft cell survivability and differentiation in this first human fetal transplant trial for spinal cord injury. A trend in the field now is to exploit the proliferative power and potency of ES cells, rather than fetal cells, for generating large numbers of dopamine neuron precursors, for example, that could be more robust and amenable to survival and integration in the very hostile environment of the compromised adult basal ganglia. But there needs to be more work on the controlled differentiation of these cells (e.g., eliminating any undifferentiated ES cells that could generate teratomas, and conferring upon them enough traits of mesencephalic A9 dopamine neurons), as well as on survivability and possible axonal growth, to successfully reestablish dopaminergic innervation and tone. Further studies are needed to refine ex vivo and perhaps in situ (via drugs that might encourage dopamine neurogenesis; see Van Kampen and Eckman 2006) methodologies that generate and apply the best dopaminergic precursor cells available from the stem cell biology field to the best animal models available (e.g., 6-OHDA and MPTP rodents and nonhuman primates, and still to be determined transgenic or knockout models that will faithfully recapitulate the course of a disease as best we know it).

The availability of ES cells offers exceptional opportunities for producing designer cells for drug screening and cell replacement as well as for combining cell and gene therapy. Again, major advantages of ES cells as a donor source include their pluripotency, the potential for virtually unlimited proliferation, their amenability to genetic modification, and the possibility to differentiate them into purified neural cell populations (Brüstle et al. 1998; Mujtaba and Rao 2002). Transplanted ES cell-derived neural precursors (ESNPs[1]), from both rodent and human ES cells (Thomson et al. 1998), incorporate widely throughout the CNS and differentiate into neurons, astrocytes, and oligodendrocytes (Brüstle et al. 1998; Zhang et al. 2001). The results of many studies indicate that primary and immortalized CNS stem cells as well as ES cell-derived neural cells can contribute to behavioral improvement when grafted into rodent models of neurodegenerative disease and of traumatic CNS injury. In addition to ES cells, SVZ-derived neural precursors and possibly even cells with attributes of differentiated neural cells—including astrotypic cells that are either low-incidence stem-like cells in adult brain gray matter or possibly amenable to dedifferentiation under the proper growth conditions (Walton et al. 2006)—might also be reasonable targets for protection and replacement protocols in CNS disease. Figures 2 and 3 document the isolation and cloning of astrotypic cells from the adult human cerebral cortex of a temporal lobectomy specimen (patient age 17 yr). After transplantation these astrotypic cells give rise to cells in the adult NOD-SCID mouse cerebral cortex and hippocampus that have many of the attributes of appropriate projection neurons (Figure 5). These adult human brain neural progenitor (AHNP[1]) cells are amenable to transplantation (Figure 5), and transduction with viral vectors (Walton et al. 2006) endows them with the ability to produce and release neurotrophic and other factors that might be important for cell protection and replacement therapeutic protocols.

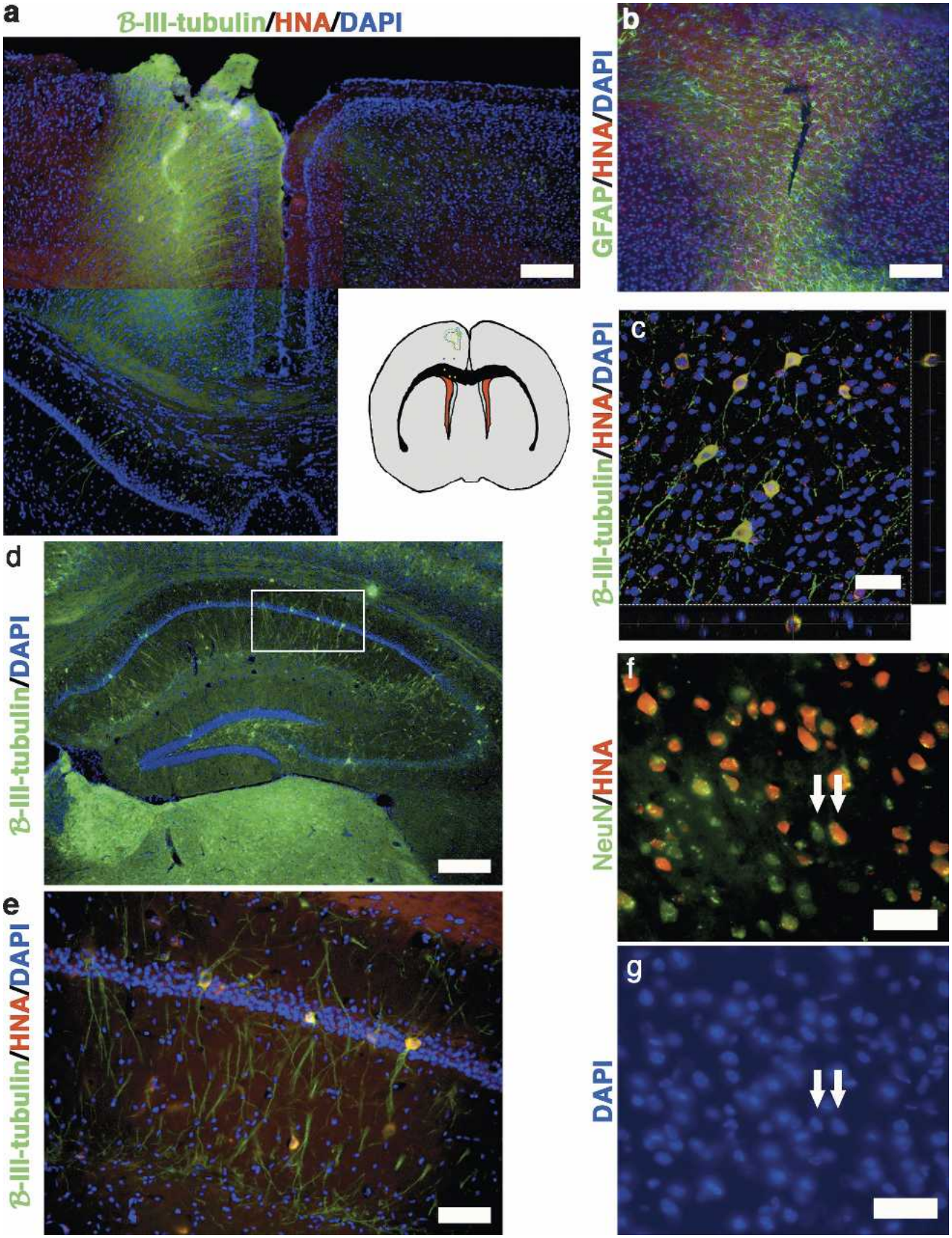


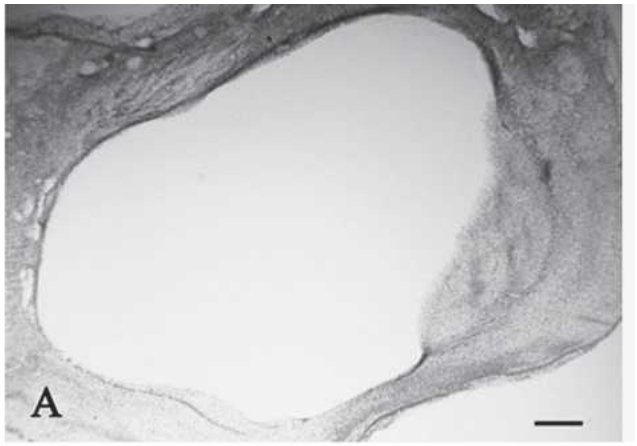
Figure 5

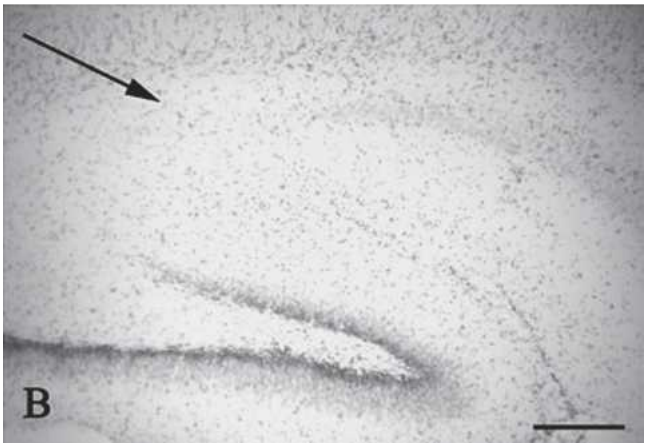
Stem cell biology and regenerative medicine also have applications in stroke. Growing numbers of studies report the injection of stem and progenitor cells from a variety of neural and nonneural sources. However, most of these studies seem to reveal positive behavioral outcomes including return of some functions that cannot clearly be tied to either neurogenesis or neuralization of the injected cells. For example, bone marrow- and cord blood-derived cell transplantations (see Peterson 2004 for review) seem to owe improvements in the ischemic CNS of these models to trophic factor(s) released by the nonneural cells that do not exhibit neural differentiation after grafting but that still seem to have a positive effect on surviving neurons and on whatever circuitry components contribute to improved movement or cognitive functions. One report has described apparent neuronal replacement from an indigenous precursor pool in the adult rat brain after a stroke (Arvidsson et al. 2002). These findings raised excitement in the field of restorative neurology about what appeared to be a cytokine-induced (SDF-1/CXCR4; Thored et al. 2006) recruitment of neuronal progenitors following cortical neuronal loss. The research supported observations of apparent self-repair attempts in the rodent cortex following injury that were initially reported by Macklis and collaborators (Magavi et al. 2000), in which newly generated cortical neurons also appeared to give rise to new long-distance axonal projections.

Thus it appears that reactive neurogenesis and attempts at neuronal replacement occur in the SVZ and dentate gyrus of the hippocampus of experimental stroke-induced rodents and primates (Lichtenwalner and Parent 2006). According to the Lindvall group (Arvidsson et al. 2002), however, most of these newly generated neurons do not survive very long. Exogenous growth factor exposure may help (Kolb et al. 2006), but a recent study (Yamashita et al. 2006) supports a notion that endogenous cell and molecular responses and interactions may be enough to support rather impressive neuronal differentiation from SVZ neuroblasts in the post-stroke adult rodent neostriatum. Growth factors and other stimulating factors (e.g., GCSF; Sprigg et al. 2006) themselves can apparently support neurogenesis (Kolb et al. 2006), and their expressions from designer nonneural cells (e.g., mesenchymal stem cells) can protect against injury in adult rodent cerebral ischemia models (Horita et al. 2006). A phase 2 randomized clinical trial in patients with subcortical stroke used cells derived from a teratocarcinoma cell line (n-tera 2 cells; Kondziolka et al. 2005) that appears to exhibit safety and possibly some feasibility for slightly improving motor function in severely debilitated patients. Chen and Chopp (2006) provide a good review of the role of cells and pharmacological approaches for "neurorestorative treatment of stroke."

But a tremendous amount of research is necessary before any cell is truly ready for widespread use in protection or repair for stroke or any other CNS disorder. For this reason, my group and others are developing new models and new cells to facilitate translation of this still basic stem cell biology to neurorepair. Two investigators in our group, Drs. Tong Zheng and Michael Weiss, have recently published a paper (Zheng et al. 2006b) on multipotent astrocytic stem cell (MASC) transplantation in a rat model of neonatal hypoxic-ischemic encephalopathy (HIE[1]; Figures 6 and 7). HIE occurs in about 20 of 1,000 full-term infants and nearly 60% of all low birth weight, premature newborns. The infants who survive have serious permanent handicaps that include cerebral palsy, learning disabilities, and seizures. The study by Zheng and colleagues showed that MASCs isolated from the mouse SVZ and the early postnatal cerebellum have the capacity to survive, migrate to the ischemic rat cortex and hippocampus (Figure 7), and undergo attempted differentiation that suggests the potential to mature into different populations of cells lost due to the ischemia. Transplantation of MASCs and other indigenous neural stem/progenitor cells seems reasonable for human pediatric HIE as the disease compromises these endogenous cells in the SVZ and they are not able to repair lost tissue as easily as their unaffected counterparts, which can be isolated from donor sources and expanded ex vivo before grafting. For example, with intraventricular transplants the MASCs seem to respond to the injury and infiltrate compromised forebrain areas (see Figure 7 and Zheng et al. 2006a). Future studies may uncover biogenic factors that help to expand and mobilize unaffected and possibly even affected MASCs and other CNS stem/progenitor cells in cases of pediatric and adult stroke and, along with behavioral modification and other forms of rehabilitation, reconstruct lost circuitries and restore lost behaviors including movement. There is a fair amount of potential for synaptic plasticity and cortical remodeling even in the adult human brain (Kaas and Qi 2004),

Figure 5 Human AHNPs transplanted into the adult NOD-SCID mouse surprisingly adopt predominantly neuronal fates. (A) Coronal section of an engrafted left hemisphere shows ß-III tubulin+ (neuronal)/HNA (human specific nuclear antigen) + donor cells adjacent to engraftment site. Schematic representation includes two-dimensional proximodistal and lateral distributions of the majority of AHNPs and ectopically migrating cells in two transplanted animals (blue and yellow). (B) Fate analysis indicates few cells adopt an astroglial identity. (C) Integrated AHNPs in the primary engraftment site adopt neuronal morphologies and neuronal immunophenotypes. (D) ß-III tubulin+ neuronal cells present within the hippocampus of engrafted animals occasionally displayed HNA (E, from boxed area in D) in CA1 and CA3, where they adopted apparent pyramidal neuron morphologies. (F,G) Single plane confocal image of cortically implanted AHNPs. HNA+ cells form mature neuronal (NeuN+) cell types, which coexist with endogenous neurons (arrowheads). Scale bars: 200 μm. Reproduced with permission from Walton NM, Sutter BM, Chen HX, Chang LJ, Roper SN, Scheffler B, Steindler DA. 2006. Derivation and large-scale expansion of multipotent astroglial neural progenitors from adult human brain. Development 133:3671-3681. Epub 2006 Aug 16. PMID: 16914491 [PubMed—indexed for MEDLINE].





Figures 6, 7 Transplantation of mouse SVZ multipotent astrocytic stem cells (MASCs) into a mouse model of pediatric hypoxic ischemic encephalopathy (HIE). **Figure 6**: Cresyl violet-stained sagittal sections of the HIE lesioned brain. (A) Cortex of a rat pup 8 days post-HIE injury. Note the marked cystic area. (B) Sections of the hippocampus demonstrated a marked loss of neurons seen in fields CA3 and CA4 of Ammon's horn (arrow) (scale bars: A = 100 μm; B = 400 μm).

therefore the next goal of restorative neurology based on stem cell biology and regenerative medicine is to prepare the reactive brain, by manipulating the molecular environment, to support reactive synaptogenesis and the reestablishment of appropriate synaptic connections (Brodkey et al. 1993). With circuitry protection and repair, it may be possible to modify even the most inhibitory of molecular environments, which we know impede axonal regeneration (Raineteau and Schwab 2001; Silver and Miller 2004), to support the growth of new neurites from newly generated neurons.

Human Chimerism, Transgenics, and Associated Issues

A recent review in the *New York Times* by Janet Maslin ("Geneticists Gone Wild; What's the World to Do?," November 28, 2006) discussed Michael Crichton's newest book, *Next*, the fictional tale of a transgenic human-chimpanzee created for a gene therapy approach for research on autism. With no progress since *The Island of Dr. Moreau* in the debates about animal-human chimerism (see J. Scott Orr, March 6, 2006, *San Diego Union-Tribune*), there is clearly a pressing need to openly discuss society's feelings and legislative urges with respect to the mixing of human and animal species for either experimentation or therapeutic application. Indeed, the ethics and morality of human-animal chimeras and transgenics are daunting, with even criminal repercussions depending on where a scientist might one day create such life forms and for what reasons. Needless to say, there should also be discussion on the other side of the issue, to consider not only under what conditions, where, and when such studies might be performed (even with therapeutic applications in mind for reducing human suffering) but also their impact on the possible suffering (at all levels of physiology, perception, and conception) of the animal side of the new life form.

It is beyond the scope of this short review to reiterate and discuss the ethical, political, and religious issues surrounding all aspects of human embryonic stem cell research, including xenografting and chimerism. Instead, readers are directed to recent articles by Irving Weissman (2004), Mark Noble (2005), and Henry Greely (2006), who have given quite a bit of thought to some of these important issues and written scholarly reviews and position papers that also reference other thoughtful treatises on the many facets of the stem cell debates.

It is nonetheless worthwhile, for this issue of *ILAR Journal*, to provide a brief discourse on the controversy of human/nonhuman chimeras. Greely (2006) discusses the utility of chimera-based models for in vivo studies of human cells and tissues in laboratory animals, just as the use of the SCID-hu mouse is very important for studying the human immune system in ways not possible with human subjects. Numerous studies have exploited humanized mice to study a variety of issues related to CNS and other somatic (e.g., vascular-related) cells (Illes et al. 2005). Greely (2006) notes that

> The NAS [National Academy of Sciences] panel recommended a flat ban on the creation of chimeras by inserting hESCs [human embryonic stem cells] into the blastocysts of nonhuman primates and, out of fear that hESCs may have led to the production of human gametes, on the breeding of any nonhuman animals into which hESCs had been introduced. . . . More broadly, it required that ESCRO [Embryonic Stem Cell Research Oversight] committees specifically review any research that would introduce hESCs into nonhuman animals, and it advised particular caution in approving experiments putting hESCs into the brains of nonhuman animals. The Canadian Assisted Human Reproduction Act, adopted in 2004, banned the creation of chimeras defined as either the insertion of a nonhuman cell into a human embryo or an embryo with cells from more than one human em-

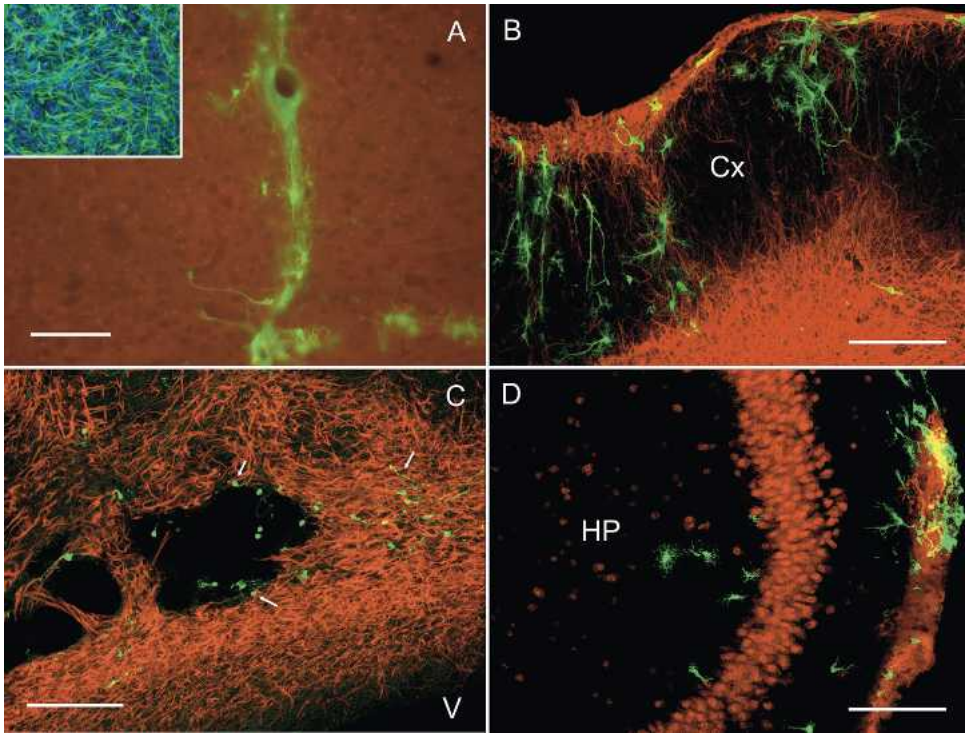


Figure 7 Survival, migration, and distribution of GFP + MASCs in the HIE-lesioned rat brain. (A) Fluorescence photomicrograph of a sagittal section through a host cortex following GFP+ MASC transplantation. Double immunolabeling for GFP (green) and neuronal β-III tubulin (red) reveal the needle track of the transplantation and the surrounding brain tissue. A large number of the GFP+ cells remained in the track. However, many cells have started to leave the injection site and spread into the surrounding area (scale bar = 100 μm). Insert shows an immunofluorescence photomicrograph of an astrocyte monolayer culture from which the donor cells were obtained. Cells were generated from the SVZ of postnatal day (P4) pups and were passaged once before analysis (13 days in culture). Most of the cells are immunopositive for the astrocyte-specific marker, GFAP (green), and no cells are labeled with neuron-specific marker β-III tubulin (red) (blue = Hoechst nuclear staining). (B) Laser confocal microphotograph shows examples of grafted cells located in the lesioned cortical areas. A sagittal section through the cortical areas 21 days post-transplant was double labeled for GFP (green) and GFAP (red). Astrocytes were highly prominent around the lesion areas and the thickness of the cortex (Cx) was significantly reduced due to cell loss. GFP+ cells appear viable and displayed mature phenotypes (scale bar = 100 μm). (C) Seven days after transplantation, donor cells were observed away from the injection site, in the lesioned cystic cortical area. These cells displayed migratory profiles characterized by fusiform-shaped cell bodies with single leading and/or trailing processes (arrows), suggesting that transplanted cells in the cortex of the HIE animals have a tendency to move toward the injured area. Green = GFP, red = GFAP, V = lateral ventricle (scale bar = 150 μm). (D) Laser confocal microphotograph shows that some of the GFP+ MASCs transplanted into the lateral ventricle attached and infiltrated into the hippocampus (HP). Some of the donor cells morphologically resembled mature neural phenotypes (scale bar = 150 μm). Green = GFP, red = NeuN. Adapted with permission from Zheng T, Rossignol C, Leibovici A, Anderson KJ, Steindler DA, Weiss MD. 2006b. Transplantation of multipotent astrocytic stem cells into a rat model of neonatal hypoxic-ischemic encephalopathy. Brain Res 1112:99-105. Epub 2006 Aug 21. PMID: 16919606 [PubMed—indexed for MEDLINE].

> bryo, fetus, or human being. . . . Senator [Sam] Brownback's proposed legislation would ban several more types of chimera. . . . Researchers and institutions will have to decide whether to follow the Brownback position or the less restrictive NAS recommendations and, either way, under what circumstances ESCROs should allow the creation of permissible human/nonhuman chimeras. . . (Greely 2006, p. 574).

A recent article from the Brivanlou laboratory (James et al. 2006) provides a profound example of applying stem cell biology toward an understanding of vertebrate, including human, morphogenesis and developmental potential of hES cells. In that study human/nonhuman chimeras revealed the ability of human embryonic stem cells (hESCs[1]) to engraft into mouse blastocysts. Despite controversy associated with studies of human/nonhuman chimeras, it is possible to generate novel data on cell-cell interactions and tissue histogenesis during embryogenesis, and the field of developmental biology has used other animal chimeras (e.g., the chick-quail) for years to provide crucial information on the origins, nature, and differentiation of various cell populations during developmental pattern formation. James and colleagues (2006, p. 97) point out that "Embryonic cell mixing and recombination experiments between related species are a traditional approach of experimental embryology,

used for more than a hundred years to understand embryonic processes at the cellular level. . . .” A goal of human/nonhuman chimeric studies seems to be the generation of animal species that can provide not only insights into histogenesis and pattern formation but also animal models for assessing donor human cell engraftment and donor cell-host interactions (e.g., without some of the complicating factors that surround the use of immunocompromised animal hosts). James and colleagues (2006, p. 100) further observe that, “with the expansion of available hESCs to include genetically diseased lines, mouse/human chimeras may allow us to elucidate the bases of disease by examining the behavior of such hESC lines in live animal models. In addition to their contribution to the basic understanding of human embryology, the advances reported here [in their report] provide a foundation for future work towards an understanding of human disease. . . .”

Despite the sensationalism and controversy associated with animal-human cell mixing studies, there is unquestionably a need to use animals as disease models and recipients to test cellular and molecular reagents that can facilitate neuropoiesis, protection, and replacement approaches for at-risk or lost CNS cells. In particular, postnatal and adult mice, and especially transgenic or knockout mice, provide invaluable insights into the efficacy of new cell and drug therapies. For the immediate future, NOD-SCID or other immunocompromised mice are probably most suitable for understanding how new stem cell transplant paradigms can lead to neural circuitry protection or repair. My laboratory’s findings about human AHNP cell transplantation in NOD-SCID mice show the exceptional transdifferentiation potential of these newly discovered human astrotypic cells into neurons, and even forebrain projection neurons, in the living forebrain of the immunocompromised host mice (Walton et al. 2006). This unexpected and surprising finding on the plasticity of a seemingly fully differentiated human brain cell would not have been possible without the use of this very special adult animal model. Knowing that these cells have this potential will enable future experimentation to explore the use of these cells as potentially very potent human therapeutics and to exploit their neural plasticity in drug-screening bioassays.

Mouse models of cancer—whether the disease is induced by genetic manipulation, chemical carcinogens, or transplantation of cancer cells—are essential for testing cell therapies and chemotherapeutic interventions. A recent study (Piccirillo et al. 2006), looking at a new molecular (BMP4) therapeutic for glioblastoma after the grafting of human glioma cells in mice, has again revealed the power of testing new cancer therapies in rodent models, although other species (e.g., the dog; Snyder et al. 2006) spontaneously develop gliomas and other primary tumors that bear remarkable likeness to those that occur in human disease. Primate models may also be appropriate for use in a regenerative medicine protocol for an investigational new drug and in phase I human clinical trials, and the FDA guidelines for bringing a new therapeutic to the clinic are fortunately quite explicit.

Conclusions

Stem cells offer great hope for treating and curing most if not all human injuries and diseases, but there is reason to believe that these cells may themselves cause tumors. This possibility is not surprising, given that, as discussed above, the job of a stem cell is to build tissue, whether normal or abnormal (after oncogenic transformation). It is therefore very important to be extremely cautious when bringing stem cell transplants, “poietic” drugs, or other regenerative medicine discoveries to the bedside.

It is the belief of this author that the regenerative medicine field should continue efforts to:

1. Discover the best stem or progenitor cell in vitro protocols for isolating, expanding, and priming such cells to facilitate their massive propagation into just the right type of neural precursor cell for protection or replacement protocols;
2. Discover biogenic factors (compounds that affect stem/progenitor cells) from HTS and other bioassay screening approaches that will encourage reactive cell genesis and appropriate functional integration in diseased or injured tissues; and
3. Establish the absolute best animal models, using both small and large animal models of disease, for the testing of new stem cell-related therapies.

In the case of protecting or replacing at-risk neurons that generate movement-related disorders, including stroke and Parkinson’s and Huntington’s diseases, an ideal protocol might involve the sequential exposure of drugs in combinations that encourage neuroprotection and selective cell genesis to thwart further degenerative processes, replace cells already lost, and promote reconnection between existing and newly generated cells. For the near future, cell transplant approaches will probably continue to be the regenerative medicine therapy of choice, using the best cells available that may come from ES, hematopoietic, or neuropoietic stem/progenitor cell sources, and possibly even from differentiated somatic cells that are amenable to dedifferentiation following exposure to the right growth factors. Ideally, rapid scientific advances in this new field of regenerative medicine will translate to new therapies and cures for all human diseases and injuries, but especially for neurological (e.g., movement and cognitive) disorders that can so dramatically affect quality of life.

Acknowledgments

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