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Stem Cells in the Central Nervous System

Ronald McKay

In the vertebrate central nervous system, multipotential cells have been identified in vitro and in vivo. Defined mitogens cause the proliferation of multipotential cells in vitro, the magnitude of which is sufficient to account for the number of cells in the brain. Factors that control the differentiation of fetal stem cells to neurons and glia have been defined in vitro, and multipotential cells with similar signaling logic can be cultured from the adult central nervous system. Transplanting cells to new sites emphasizes that neuroepithelial cells have the potential to integrate into many brain regions. These results focus attention on how information in external stimuli is translated into the number and types of differentiated cells in the brain. The development of therapies for the reconstruction of the diseased or injured brain will be guided by our understanding of the origin and stability of cell type in the central nervous system.

Definition of the processes that shape the cellular makeup of the central nervous system (CNS) has relied heavily on three distinct procedures: fate mapping, tissue culture, and transplantation. These traditional tools of embryologists have been significantly improved by the recent incorporation of advanced molecular methods. Fate mapping of neuronal precursors in vertebrates points to the existence of multipotential cells that are precursors to both neu-

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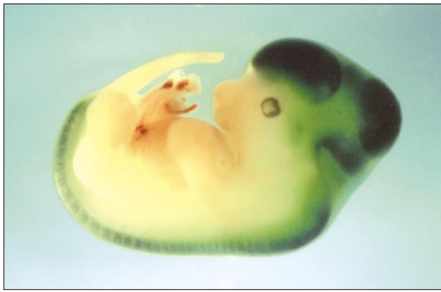
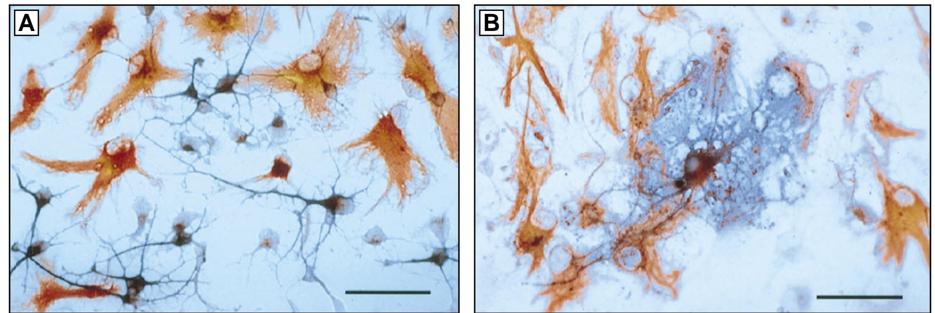


Fig. 1. A transgenic mid-gestation mouse fetus showing the expression (blue) in CNS stem cells of a reporter gene under control of 750 base pairs of the second intron of the nestin gene. The approach is described in detail in (*9*).



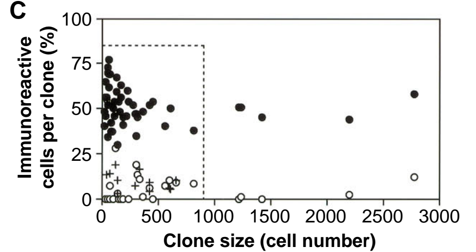


Fig. 2. The differentiation of adult CNS stem cell clones: (**A**) neurons (blue) and astrocytes (red); (**B**) oligodendrocytes (blue) and astrocytes (red). Scale bars: 25 μm. (C) The proportion of cells of different types in fetal stem cell clones: (●) neurons, (○) astrocytes, and (+) oligodendrocytes. The proportion of neurons that differentiates in a clone is constant, independent of clone size. The same proportion of neurons differentiates in adult and fetal stem cell clones. Data taken from (*20*).

rons and glia (*1*). However, this approach does not necessarily reveal the full proliferation and differentiation capability of the cells. In vitro and in vivo manipulations must be used to test the developmental potential of a cell. Tissue culture and transplant techniques, developed in vertebrate systems (*2*), have generated important data on the potential of neural cells (*3*).

Defining a Stem Cell

To be considered a stem cell in the CNS, a cell must have the potential to differentiate into neurons, astrocytes, and oligodendrocytes and to self-renew sufficiently to provide the numbers of cells in the brain. The term "progenitor" refers to a cell with a more restricted potential than a stem cell. "Precursor" is a less stringent term that refers to any cell that is earlier in a developmental pathway than another.

The complete cellular lineage of the nematode *Caenorhabditis elegans* has been described (*4*) and is an influential instance of the power of morphological analysis to define precursor-product relations in vivo. However, in the CNS of mammals, there are too many cells for each to be followed individually. The problem is similar to the technical difficulties biochemists faced in defining metabolic pathways. Without access to pure precursor, it was difficult to establish the catalytic step actually performed by a given enzyme. When this hurdle was overcome, it was recognized that enzymes perform discrete chemical steps, ultimately giving rise to the important concept of one gene–one enzyme (*5*). Similarly, to understand the developing brain, we need to purify the precursor cell types and define their transitions into differentiated progeny. Early work revealed that fetal cells removed from the developing brain and placed in vitro could give rise to differentiated neurons (*6*). For the most part, these neurons were derived from cells that did not divide in tissue culture, although cells that did divide could acquire some features of immature neurons (*7*).

The intermediate filament nestin is a major cytoskeletal protein in neuronal precursors in the mammalian CNS (*8*). Nestin is first detected at the earliest steps in neural plate induction (*9*), and most cells in the neuroepithelium are nestin-positive before neurogenesis (*10*) (Fig. 1). Coincident with their exit from the cell cycle, neurons down-regulate nestin and express distinct intermediate filaments. This transition has also been observed in vitro where precursor cells proliferate and differentiate into neurons (*11–16*) and glia (*17*).

Confirming the results of in vivo fate mapping, lineage experiments in vitro show that neurons and glia can be derived from a common fetal precursor cell (*12, 13, 15, 16, 18–20*) (Fig. 2). The adult nervous system also contains multipotential precursors for neurons, astrocytes, and oligodendrocytes (*13, 16, 18–20*). Cultured cells from both the fetal and adult CNS that have proliferated in vitro can differentiate to show morphological and electrophysiological features characteristic of neurons: regenerative action potentials and synaptic structures (*16, 21*) (Fig. 3). These data show the multipotential nature of cells derived from the CNS.

Quantitative studies have established the homogeneity and stability of multipotent cells derived from the fetal brain (*20*). In vitro these cells divide daily and efficiently generate neurons and glia for at least the first month of culture. These multipotent cells proliferate sufficiently in vitro to

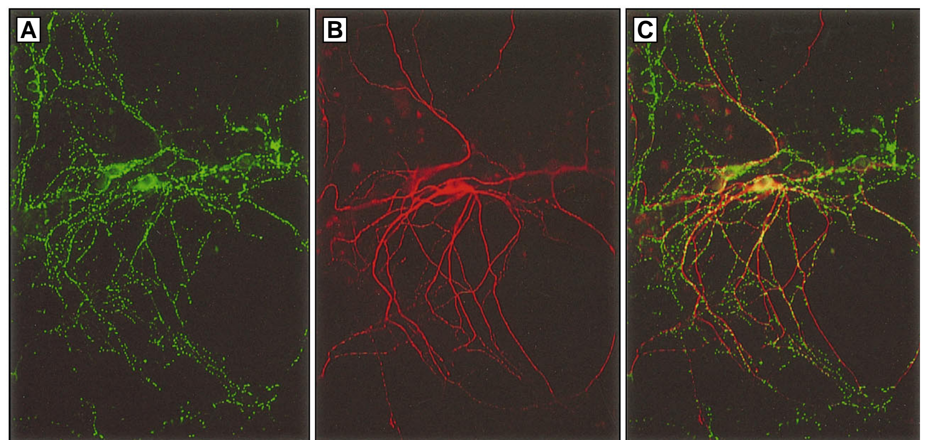


Fig. 3. Neuronal differentiation of CNS stem cells derived from the embryonic day-16 hippocampus. Cells were expanded for 16 days in the presence of bFGF followed by 21 days of differentiation in the presence of BDNF (20 ng/ml). (**A**) Staining with antibody to synapsin (green), (**B**) staining with antibody to MAP2, and (**C**) the two images superimposed. Synapsin is concentrated in presynaptic terminals, and MAP2, in dendrites. The culture and staining conditions are similar to those reported in (*21*).

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account for the large numbers of cells present in the mammalian brain at birth (*10*). These cells can be considered to be stem cells because they fulfill the criteria of multipotency and self-renewal. Asymmetric division, which is sometimes considered to be a property of stem cells (*2*) and may actually occur in the neuroepithelium (*22*), does not appear to be necessary in cultured CNS stem cells (*20*).

Response Mechanisms and Transitions in Vitro

The extraordinary diversity of the adult vertebrate nervous system is generated from a sheet of epithelial cells over a period of several days. Precise numbers of neurons, astrocytes, and oligodendrocytes differentiate in successive waves. The spinal cord, formed from the caudal region of the neural tube, is one of the first sites of neuronal differentiation. Basic fibroblast growth factor (bFGF) is one mechanism that defines rostro-caudal identity in the neural tube (*23*). Neuronal differentiation in the dorsoventral axis is a response of uncommitted cells to successive extracellular signals (*24*). Sonic hedgehog and members of the transforming growth factor–β (TGF-β) family influence ventral and dorsal features of development in the caudal neural tube. These signals are used in several cellular contexts. For example, members of the TGF-β family influence segment-specific apoptosis in the neuroepithelium (*25*), astrocyte maturation (*26*), the differentiation of peripheral nervous system stem cells (*27*), and dorsoventral differentiation in the CNS (*24*). These diverse effects emphasize that the action of extracellular ligands depends on the integration of multiple signals by a specific responding cell.

Cell-autonomous mechanisms may also contribute to the generation of cell types in the nervous system. In the hematopoietic system, cell-autonomous stochastic processes are thought to generate all of the mature cell types, and the specificity of differentiation is a consequence of selective mechanisms (*28*). In such a system, specificity is obtained as a consequence of signals acting selectively only after the events that generate the different cell types. There is clear evidence for cell death in the neural tube (*29*) and growing knowledge of extra- and intracellular signals that mediate cell death (*30*). The high rates of apoptosis during neural development are consistent with an important role for selective mechanisms in the CNS.

Instructive mechanisms also occur in both the peripheral (PNS) and central nervous systems. Glial growth factor, a member of the heregulin-neuregulin class of factors, acts instructively on PNS stem cells to direct them to a Schwann cell fate (*31*). Bone morphogenetic proteins (BMP) 2 and 4 stimulate neurogenesis, and TGF-β1 generates smooth-muscle cells from the PNS stem cell (*27*). In the CNS, ciliary neurotrophic factor (CNTF) acts instructively on the multipotential stem cell, directing it to a committed astrocytic fate (*20*).

It has previously been shown that CNTF induces astrocytic differentiation in O2-A cells (*32*). In vitro, CNS stem cells rapidly and efficiently differentiate into astrocytes in the presence of CNTF (*20*). CNTF is not a mitogen for these cells, and a transient exposure (48 hours) to CNTF, even in the presence of mitogen, switches the differentiated state of more than 98% of the uncommitted stem cells. These data suggest that, in the absence of significant cell death, stimulating the Jak-stat system (the effector of CNTF) instructs the stem cell to become an astrocyte. In a recent study, BMPs promoted astrocytic differentiation from cells that had been expanded in vitro in the presence of epidermal growth factor (*26*). It will be interesting to establish whether BMPs and CNTF act through a common pathway at the same stage of astrocyte differentiation.

Thyroid hormone (T3) is also an instructive factor causing stem cells to become lineage-restricted progenitors for oligodendrocytes (*20*). Interestingly, CNTF and T3 are both differentiation and lineage-restriction factors. The differentiation of peripheral and central stem cells can be achieved without selection by the instructive action of extracellular signals. However, it seems likely that a combination of instruction and selection is used in vivo to precisely regulate precursor-product transitions at the cellular level.

The importance of selective mechanisms acting on a defined precursor cell type in brain development is best illustrated by studies on the differentiation of oligodendrocytes. There is evidence from the optic nerve for the existence of a bipotential progenitor in vitro for oligodendrocytes and type-2 astrocytes, the O-2A cell (*33*). In addition, the differentiation of this precursor cell could be controlled by manipulation of extracellular signals. Once the properties of this cell had been established, it became clear that a similar cell existed in the adult optic nerve (*34*). In tissue culture, O-2A cells respond to several factors [bFGF, platelet-derived growth factor, CNTF, neurotrophins, and T3], and many of these factors also act in vivo to increase oligodendrocyte number in the optic nerve (*35*). These in vivo data show that the availability of growth factors is limiting and that cell death is important in regulating oligodendrocyte numbers.

This summary indicates that simple ligands can regulate in vitro the transitions between stem cells and the three major cell types of the adult brain. However, it is not clear how many cell states exist in addition to stem cells and committed progenitors for astrocytes and oligodendrocytes. In some cases precursor-product transitions have been defined, but there are still many aspects of cell-type origins that are unclear and may be advanced by further work in vitro. Epidermal growth factor (EGF) and bFGF have both been used as mitogens to expand CNS stem cells, but EGF may not be the optimal choice for a stem cell mitogen, as there is evidence that EGF favors glial differentiation. In vitro EGF is a stem cell mitogen and a differentiation factor for astrocytes but not a lineage restriction factor, suggesting that the commitment event is distinct from the differentiation mechanism (*20*). However, the in vivo overexpression of EGF receptor may induce a fate shift from neurons to glia rather than simply promote astrocytic differentiation (*36*). It is clearly necessary to define the fundamental biochemical differences between lineage restriction in stem cells and differentiation of progenitor cells.

Another important unresolved question is whether there are proliferating cells capable of giving rise to specific kinds of neuron. There is evidence for a cell of this type in the postnatal cerebellum, but it is not clear whether a committed neuronal progenitor occurs in other brain regions (*37*). The events that generate the pluripotent CNS stem cell from an earlier totipotent embryonic stem cell can also be analyzed in vitro, because embryonic stem cells differentiate through a nestin-positive state to form synaptically active networks of central neurons (*38*). The routine differentiation of functional neurons from propagated stem cells would permit detailed analysis of how early steps in neurogenesis influence later stages of neuronal differentiation. The challenge is to set up experimental systems where the differentiation events of interest can be measured efficiently.

Space and Time

The cortical neuroepithelium is a highly polarized structure. Precursor cells divide at the inner (ventricular) surface of the neural tube, and immature neurons migrate away from the ventricle to specific layers. As different neurons become postmitotic in sequence, their laminar location is a function of the time when the neuron differentiated. Transplants in ferret cortex show that appropriate, layer-specific neuronal differentiation occurs when cells derived from an early time are moved to a later stage (*39*).

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Conversely, late neuronal precursors transplanted to an earlier stage host do not contribute efficiently to early neuronal fates but rather exhibit laminar positions appropriate for late-generated neurons (*40*). This evidence supports a model where a neuron becomes committed to a particular laminar fate in the ventricular zone at the time of withdrawal from the cell cycle. Thus, it is the timing of the exit from the cycle that is thought to implement two distinct commitment events. In this scenario, the postmitotic neuron is locked into a specific fate, and the remaining precursors are also irrevocably changed.

It is not known what specifies the regional identity of the different areas of the CNS. There are complex patterns of expression of both cell surface signals and transcriptional regulators in the developing neuroepithelium long before neurons themselves differentiate (*41*). But there could be different stem cells for different brain regions. Gene deletion experiments in mice illustrate that whole sections of the brain can be eliminated with relatively little perturbation of the development of adjacent brain regions (*42*). Although these results are startling, they do not establish whether neuronal precursor cells are irreversibly committed to distinct regional fates. To establish commitment, we must give cells an opportunity to choose another regional fate. In the developing chick, a duplication of a brain region can be obtained by the local application of FGF8 (*43*). This result suggests that single factors are sufficient to bias the differentiation cascade and establish major regional features of the CNS.

The rhombomeres of the hindbrain are a good example of the compartmental arrangement of the neuroepithelium (*44*). Although it was first thought that cells were prohibited from crossing the boundaries between rhombomeric compartments, fate mapping in vivo now suggests that cells do move from one compartment to another at a low frequency (*45*). In other brain regions, neuronal precursors also migrate over great distances (*46*). When the location of rhombomeres was altered by tissue grafts, rhombomere-specific *Hox* gene expression was respecified by as yet undefined anterior-posterior control systems (*47*). In these transplant experiments, pieces of tissue were rearranged, making it hard to interpret the responses of single cells. It will be interesting to directly test the plasticity of isolated rhombomeric cells by transplanting dissociated cells from one rhombomere to another.

Grafting experiments with cell lines from the hippocampus support a model in which local signals in the neuroepithelium at the time of neurogenesis give rise to region-specific neuronal subtypes. Immortalized nestin-positive hippocampal cells transplanted to the developing cerebellum differentiated into typical cerebellar neurons (*48*). Transplants of primary striatal cells into the developing cerebral cortex also showed a switch to the locally appropriate fate (*49*), suggesting that the plasticity in cell fate shown with immortal cells was not an artifact of immortalization. In conceptually similar experiments, primary cerebellar cells derived from mice expressing the *lacZ* reporter gene under a neuron-specific promoter were grafted into the hippocampus of neonatal rats or wild-type mice. The grafted cells acquired morphological and immunohistochemical features of hippocampal granule neurons (*50*). The grafted and host neurons also showed kinetics of induction identical to those of the immediate early gene *c-fos* after intraperitoneal injection of neurotransmitter agonists and antagonists (*50*). These data suggest that immortal and primary neuroepithelial precursor cells grafted to new sites generate region-specific neurons in response to local cues.

A major limitation of postnatal transplantation studies was that heterotopic neuronal integration occurred efficiently only when donor cells were introduced into the few sites that continued to generate neurons in the newborn animal. This limited spectrum of accessible regions was dramatically increased by transplanting neural cells across the uterine wall into the embryonic mammalian brain (*49, 51, 52*). When genetically labeled mouse telencephalic neuroepithelial cells were simply deposited in the ventricles, large numbers of grafted cells were subsequently found incorporated into many sites in the host brain. The transplanted cells migrated in accordance with known pathways and incorporated into telencephalic, diencephalic, and mesencephalic regions (*52*). Surprisingly, cells derived from the dorsal and ventral forebrain incorporated into homotopic and many heterotopic brain regions in a similar fashion. After migration, the cells acquired morphological and antigenic features appropriate for neurons in their new environment (Fig. 4). The fact that striatal precursors can give rise to cortical, thalamic, and even tectal neurons illustrates that the regional heterogeneity of the brain results primarily from extracellular signals acting on precursors during neuronal migration and differentiation. These results indicate that the activation of different signaling pathways in uncommitted stem cells generates the spatial heterogeneity of neurons seen in the CNS.

Stem Cells and Disease in the Adult Nervous System

It is important to define the types of precursor cells that give rise to the neurons generated in the adult CNS (*53, 54*). Cells from the adult brain proliferate and differentiate into neurons and glia in tissue culture (*13, 14, 16, 55*) with the same efficiency for neuronal differentiation as found in fetal stem cells and the same responses to extracellular ligands (*20*). For example, 50% of the cells differentiate into neurons, and glial differentiation is strongly enhanced in response to CNTF and T3 in fetal and adult stem cells. Thus, similar general mechanisms control the differentiation of stem cells from fetal or adult brain. In contrast to this apparent homogeneity in vitro, the behavior of cells in the adult proliferative zones in vivo is more difficult to define. Nevertheless, precursor cells in the adult forebrain have been intensely studied (*19, 54, 56*). The proliferation of these cells can be stimulated by the direct application of mitogenic growth factors in vivo, and in animals treated in this way, proliferating cells in the subventricular zone differentiate into neurons and glia (*57*). However, in vivo less than 3% of the proliferating cells labeled with bromodeoxyuridine differentiate into neurons. The discrepancy between the efficient neuronal differentiation of adult stem cells in vitro and their inefficient differentiation in vivo is a critical but unresolved question for the field. Thus, the lack of differentiating neurons may not be a consequence of the lack of cells with the appropriate potential but rather a function of the signaling environment in the adult brain. However, a careful analysis of adult stem cells has only just

Fig. 4. Genetically labeled cells differentiate into hippocampal CA1 pyramidal neurons. The donor cells were derived from the embryonic day-14 cortical neuroepithelium of a transgenic mouse carrying a *lacZ* reporter gene. They were placed into the telencephalic vesicles of an E18 rat, where they incorporated into the host hippocampus and differentiated into granule and pyramidal neurons. The grafted cells can be identified by the blue *lacZ* signal. Data taken from (*52*). Scale bar: 50 μm.

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begun, and we cannot yet rule out cell-autonomous restrictions that make the adult stem cells distinct from their fetal counterparts.

There is traditionally a close interaction between fundamental and clinical goals in the study of stem cells (*58*). The identification of extracellular proteins that regulate the differentiation of multipotent cells derived from the adult brain has implications for therapies targeted at neurodegenerative disease. The increased interest in extracellular signals acting on plastic cells during development fits well with the massive effort mounted in the biotechnology community to develop treatments for neurodegenerative disease based on the delivery of neurotrophic proteins. In vitro neuronal survival assays were often used in the initial identification of neurotrophic factors. These factors were then rapidly tested in animal models of neurodegenerative disease. The long-term delivery of proteins in the brain is a major goal in gene therapy. Transplantation of cells engineered to produce growth factors shows the potential of grafted cells as vectors for protein delivery (*59*). However, the complexity of neurotrophic signals still challenges the technology for gene manipulation and protein delivery in the CNS. There has been encouraging progress in using cell lines derived from the neuroepithelium rather than fibroblasts as cellular vectors in models of CNS disease. Neuroepithelial cells integrate in the host more readily than fibroblasts. This feature is an advantage for distributing a soluble ligand more widely in the diseased brain (*60*) or correcting a general biochemical deficit in the CNS (*61*).

It is possible to generate many different immortal cell lines from the developing CNS. These cells can express characteristics of stem cells (*48, 62*), neurons (*63, 64*), or glia (*17, 65*). Immortalized neuroepithelial stem cells can show extensive morphological differentiation into neurons when they are grafted into the developing (*48, 62*) or adult brain (*64*). The differentiation of genetically labeled immortal cells into neurons when implanted into the adult brain is notable because it hints that neuronal replacement in the adult is not only possible but might become simple. In most cases, immortalization has been achieved by incorporating oncogenes into a primary cell, which is, of course, not advisable for actual clinical use. However, the CRE-loxP system may be useful for removing the immortalizing oncogene before implantation (*66*).

More recently, the field has shifted away from the use of oncogene-immortalized cells toward the grafting of primary cells expanded in vitro. An example of this development is an experiment suggesting that primary adult cells derived from the hippocampus and cultured for long periods in vitro can still differentiate into neurons when re-implanted into the migratory pathway used to replenish neurons in the adult olfactory bulb (*67*). Although this field is still technically demanding, these and other results discussed here suggest that further experimental work should be directed at ambitious cell therapies based on both primary and immortal cells derived from the neuroepithelium. Clinical trials show that neuron replacement therapies for neurodegenerative diseases, such as Parkinson's and Huntington's disease, are feasible (*68*). Neural grafting is currently limited by a number of factors, including the lack of suitable donor material and the full integration of the grafted cells. In vitro expansion and manipulation of cells from the neuroepithelium will provide a range of well-characterized cells for transplant-based strategies for neurodegenerative disease (*69*). Experimental grafts in animal models suggest that the integration of grafted neurons into the circuitry of the host may be possible (*50, 52, 62, 64, 69*). Appropriate pretreatment of the host brain may be required for efficient neuronal differentiation by grafted precursors (*70*). For clinical applications, cell culture offers an important opportunity to use sophisticated genetics in cell-based therapies for neural disease.

The clinical significance of stem cell biology extends beyond cell-based therapies. The dynamics of cell organization is also critically relevant to a systematic understanding of CNS tumors and of physical injury to the brain. Two examples of nestin expression in the adult brain illustrate this point. In addition to being expressed in adult stem cells, nestin is also found in CNS tumors (*71*) and reactive astrocytes (*72*). These observations raise the interesting question of the extent of similarity between these nestin-positive cells and CNS stem cells. The proliferation and migration of CNS tumor cells are their two most damaging features. It is tempting to speculate that the self-renewing cell in a CNS tumor is similar to the stem cells found in the fetal and adult CNS.

These examples illustrate the much more general point that there will be a wide clinical impact resulting from increased knowledge of the mechanisms that control the transitions between cell types in the adult CNS. The clear-cut properties of dissociated CNS stem cells in culture show that in vitro technology can be used to define, at the cellular and molecular levels, the steps in fate choice. The presence in the adult of multipotential cells similar to the fetal stem cell emphasizes the importance of extracellular signals acting on stem cells throughout the mammalian life cycle. As our understanding of the nature of these signals grows, therapies will be developed in which the responses of normal and diseased stem cells will be manipulated to clinically useful ends.

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Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues

Darwin J. Prockop

Marrow stromal cells can be isolated from other cells in marrow by their tendency to adhere to tissue culture plastic. The cells have many of the characteristics of stem cells for tissues that can roughly be defined as mesenchymal, because they can be differentiated in culture into osteoblasts, chondrocytes, adipocytes, and even myoblasts. Therefore, marrow stromal cells present an intriguing model for examining the differentiation of stem cells. Also, they have several characteristics that make them potentially useful for cell and gene therapy.

Because circulating blood cells survive for only a few days or months, hematopoietic stem cells (HSCs) in bone marrow must provide a continuous source of progenitors for red cells, platelets, monocytes, granulocytes, and lymphocytes (*1*). However, bone marrow also contains cells that meet the criteria for stem cells of nonhematopoietic tissues. The stem-like cells for nonhematopoietic tissues are currently referred to either as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells (MSCs), because they appear to arise from the complex array of supporting structures found in marrow.

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Multipotentiality of MSCs

The presence of stem cells for nonhematopoietic cells in bone marrow was first suggested by the observations of the German pathologist Cohnheim 130 years ago (*2*). Cohnheim studied wound repair by injecting an insoluble analine dye into the veins of animals and then looking for the appearance of dye-containing cells in wounds he

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