Introduction to Neural Stem Cells

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Abstract*—**Neural stem cells self-renew and give rise to neurons, astrocytes and oligodendrocytes. These cells hold great promise for neural repair after injury or disease. However, a great deal of information needs to be gathered before optimally using neural stem cells for neural repair. This brief review provides an introduction to neural stem cells and briefly describes some advances in neural stem-cell biology and biotechnology. (Stroke*. 2007;38[part 2]:810-816.**)

Key Words: basic science ■ brain ■ brain recovery ■ stem cells

One of the fundamental concepts of neural repair lies in the replacement of cells that are lost as a result of disease or injury. Numerous investigators have used a variety of means to replace cellular elements in the brain, including transplantation of fetal tissue, primary cells derived from a number of different structures and transformed or genetically engineered cell lines. With each of these modalities numerous problems exist, including access to sufficient numbers of cells, lack of specificity of the repair strategy, immunologic rejection, and, most importantly, lack of efficacy. The discovery of neural stem cells, however, has led to a renewed hope in cellular replacement in the central nervous system (CNS) after stroke or other insult.

Neural stem cells have the capacity to self-renew and produce the 3 major cell types of the CNS. Although the studies of Reynolds, Weiss and colleagues definitively isolated neural stem cells for the first time,[1,2] a number of prior studies set up this discovery. For example, the in vitro studies of Raff and colleagues demonstrated that a common progenitor existed for oligodendrocytes and astrocytes.[3] In 1992, Reynolds, Weiss and colleagues demonstrated that cells could be isolated from the CNS of adult and embryonic mice and propagated in the presence of epidermal growth factor to give rise to large spheres of cells that they termed "neurospheres." These neurospheres possessed neurons and glia, but largely consisted of cells expressing the intermediate filament previously associated with neuroepithelial cells, nestin. They showed that an entire neurosphere could be generated from a single cell and that this neurosphere could be subsequently dissociated to produce a new neurosphere that also contained neurons and glia. Thus, these neurosphere-producing cells had the properties of a stem cell: they were self-renewing and multipotent.

In another, often parallel line of discovery, the potential significance of neural stem cells in the adult CNS was established. For a number of years, it was held that there was no neurogenesis in the adult vertebrate brain. The studies of Nottebohm and colleagues[4] demonstrated that adult male songbirds had a robust period of neurogenesis during the spring mating season. As early as 1969, neurogenesis in the adult rodent olfactory bulb was described,[5] with confirmation of this work published in 1977,[6] although these studies were largely ignored. However, in 1993 the studies of Luskin[7] and Lois and Alvarez-Buylla[8] and colleagues clearly demonstrated that the ongoing proliferation of cells in the adult rodent subventricular zone (SVZ) resulted in new neurons within the olfactory bulb. Although not completely proven, current theory holds that within the adult SVZ, a relatively quiescent stem cell gives rise to rapidly proliferating progenitors, which then ultimately give rise to neuronal precursors that migrate into the olfactory bulb to form granule cells and some periglomerular interneurons.[9] In addition to the olfactory bulb, new neurons are formed in the adult mammalian hippocampus where new dentate gyrus granule cells are regularly added.[10]

The Adult Neural Stem Cell

A great deal of attention has been given to precisely identifying the adult neural stem cell. Early work suggested that ependymal cells lining the lateral ventricle were adult neural stem cells.[11] However, a number of studies have called these findings into question. Work by Doetsch et al provided strong evidence that a GFAP-expressing cell in the subventricular (subependymal zone) is capable of replenishing the SVZ after ablation and give rise to neurons in vitro and in vitro.[12] Laywell et al[13] found that, in vitro, GFAP containing cells grown as traditional "astrocyte" cultures can form neurospheres and give rise to neurons as well as glia. Sofroniew and colleagues (including us)[14,15] demonstrated that the vast majority, if not all neurosphere-forming cells derived from the murine forebrain are GFAP positive. Additionally, they found that virtually all postnatally born neurons are ultimately derived from GFAP positive cells.[16] Recently, similar cells were demonstrated in the adult human SVZ, although a

Received September 6, 2006; final revision received December 5, 2006; accepted December 6, 2006.

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***Stroke* is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000255757.12198.0f**

clear rostral migratory stream could not be identified.[17] Taking these studies together, there is very strong evidence that adult forebrain neural stem cells are GFAP positive and located in the SVZ. However, it is still possible that a relatively small number of neural stem cells are ependymal cells which may give rise to the GFAP positive cells.

Neural Stem Cells in the Developing Brain

Neural stem cells in the developing brain appear to bear important differences from those in the adult brain. Before embryonic day 15 in the mouse, there are few, if any, GFAP positive cells within the brain.[18] Because cells meeting at least some of the criteria of neural stem cells can be cultured from any embryonic stage after neural tube formation, and possibly before, these cells must be different to some degree from adult neural stem cells. There is no evidence of a quiescent, slowly dividing neural stem cell during early brain development. This makes sense because a great deal of stem cell proliferation would be required to generate the large numbers of cells in the mammalian brain. Furthermore, it is apparent that neural stem cells change their characteristics during development. In vivo, a wave of neurogenesis precedes gliogenesis. At least some of these tendencies are retained in vitro. Neural stem cells cultured from early to mid-gestation give rise to more neurons than those cultured at later periods, a property that appears to be cell-intrinsic.[19] These properties, however, are subject to manipulation. Deletion of the tumor suppressor gene, *PTEN*, for example, allows neural stem cells derived from embryonic mouse cortex to retain their neurogenic capacity for longer periods in culture.[20,21]

As in the adult, the cellular source for neural stem cells in the developing brain is being intensively studied. Nestin is an intermediate filament expressed by the neuroepithelial cells of the neural tube.[22] Nestin-positive cells make contact with the ventricular surface and have radially oriented processes that make contact with the pial surface. These cells become radial glia, which have long been known to play key roles in radial migration of nascent neurons. However, many studies have now clearly demonstrated that radial glia of the developing brain function as neural stem cells, giving rise to neurons, glia and other neural stem cells.[23,24]

In addition to CNS (brain and spinal cord) neural stem cells, a distinct population (or populations) of stem cells exists that forms neural crest-derived elements, including neurons, Schwann cells, smooth muscle cells and pigmented epithelium.[25,26] These neural crest stem cells may ultimately be derived from CNS stem cells, but have numerous properties that distinguish the 2 populations. Neural crest stem cells will not be considered further in this brief review.

Isolation and Culture of Neural Stem Cells

Neural stem cells were initially cultured as floating neurospheres in the presence of epidermal growth factor (EGF) from adult and embryonic murine forebrain.[1,2] Using this culture method, brain tissue is dissociated and suspended in a relatively simple medium in the presence of hormonal supplements (eg, N2; B27) and mitogen. Following initial reports of the culture of NS as neurospheres, stem cells were cultured from the adult murine hippocampus as monolayers in the presence of basic fibroblast growth factor (bFGF).[27] It is now clear that neural stem cells can be cultured from virtually any region of the CNS that contains a germinal center as either monolayers or neurospheres. In general, most studies have shown the neural stem cells derived from the brain respond to either bFGF or EGF. However, NS cultured from the early embryonic forebrain do not respond to EGF until they acquire EGF receptors at later stages of development in vitro or in vivo.[28] Additionally, it has also been reported that spinal cord neural stem cells will only respond to a combination of bFGF and EGF.[29] However, our unpublished studies suggest that this is not the case, but that at any age beyond E14, murine spinal cord neural stem cells respond to either growth factor alone, although the combination of EGF and bFGF is more efficacious. On E11, however, spinal cord neural stem cells do not proliferate in the presence of EGF alone, but EGF enhances the action of bFGF.

Although either EGF or bFGF can be used to induce the proliferation of neural stem cells, these 2 growth factors are not completely interchangeable. It appears that propagating neurospheres in the presence of EGF alone (or other factors that stimulate the EGF receptor) results in a greater degree of commitment to glial lineages, whereas bFGF allows for a greater extent of neurogenesis under most circumstances.[30]

Regardless of the methods by which neural stem cells are induced to divide in vitro, they must possess the key properties of self-renewal and multipotency. Self-renewal, the ability of a neural stem cell to produce another neural stem cell can be symmetric—where 1 neural stem cell produces another neural stem cell as well as a more differentiated or committed cell—or symmetric, where a neural stem cell produces 2 copies of itself. Because of a lack of specific markers, it is somewhat difficult to precisely determine that a neural stem cell has produced a true copy of itself. Often, self-renewal is operationally defined as the degree to which a neural stem cell can give rise to colonies (neurosopheres or attached) that differentiate into neurons, astrocytes and oligodendrocytes. This requires culture under clonal conditions, to ensure that any group of cells being examined is derived from a single cell. Ensuring such clonality is difficult because the culture conditions to generate individual cells are quite harsh and floating cells tend to aggregate. One can grow neurospheres in a small well after cell sorting or serial dilution to place only one cell in a well. However, performing analysis on these cultures can be quite labor-intensive and the number of neurospheres that one obtains is often insufficient for data analysis. Therefore, in our laboratory, we often perform largescale culture analysis under floating conditions in tissue cultures. In floating cultures, seeding cells at a density of <1 cell per microliter in a T25 or T75 flask allows one to state that the vast majority of neurospheres observed were generated from a single cell. One has to be quite cautious in their methodology, however, because placing cells at the same density in 6 well tissue culture clusters dramatically reduces the percentage of neurospheres that are truly clonal (Kelly TK, et al, unpublished observations, 2006). Once clonal conditions are ensured, one can estimate the number of neural stem cells and, hence, their self-renewal

capacity after multiple passages. Theoretically, a true neural stem cell should be capable of infinite or extensive self-renewal. Very few studies have performed such analysis on neural stem cells derived from adult or embryonic CNS. However, it appears that cells propagated from the SVZ adjacent to the lateral ventricle at most stages in development can extensively self-renew. On the other hand, cells cultured from the postnatal brain, in areas outside of the striatal SVZ (such as the neocortical germinal zone) appear to have a more limited capacity for self-renewal and should probably be deemed multipotent progenitors rather than true neural stem cells.[31] One population of such limited multipotent progenitors exists outside of the boundaries of the neuroepithelial ventricular or SVZs. Roy et al[32] showed that murine and human CNS white matter contains a population of progenitors that is capable of generating neurospheres and producing neurons and glia.

By its nature, the culture of neural stem cells as neurospheres takes a complex mixture of cells and enriches for neural stem cells. This enrichment has proven highly useful in a number of studies of the molecular and cell biology of neural stem cells, but is clearly not a true purification. As such, a number of investigators have attempted to purify neural stem cells using cell sorting under a variety of conditions. Ritze et al[33] used cell size as well as lectin-binding ability to enrich for murine neural stem cells directly from the brain. Others have used the basic ability of stem cells to exclude fluorescent dyes to enrich for neurosphere-forming capacity.[34] Enrichment based on antigenic properties has also been demonstrated using the CD133 (AC133) antigen in fetal human samples[35] and the Lewis or LeX antigen in murine samples.[36] Still another method has used promoters of genes that are highly enriched in neural stem cells to drive the expression of green fluorescent protein before fluorescence activated cell sorting.[37,38] All of these methods appear to have use and enrich neural stem cells to a greater extent than simply culturing the cells, but all have limitations. First, these methods provide enrichment rather than purification. Also, because of the methods that are used there is inevitable cell loss, often diminishing the yield of neural stem cells to make subsequent studies difficult. To date, there is no set of markers that precisely identifies a neural stem cell and distinguishes it from other more limited progenitors, precursors or differentiated cells. Therefore, the formal identification of cells as neural stem cells still requires the functional demonstration of self-renewal and multipotency.

As is the case for any other cell, culturing neural stem cells changes their properties, so one cannot assume that studies of gene expression or cell fate on cultured cells necessarily implies similar mechanisms in vivo. For example, although spinal cord neural stem cells can be cultured for extensive periods and retain expression of key spinal cord genes, they lose characteristic gene expression of the dorsal or ventral cord from which they are originally derived.[39] It has also been demonstrated that, at least under some conditions, cultured neural stem cells will transform in to tumor-like cells.[40]

Neural Stem Cell Biology

It is well outside the scope of this review to summarize the thousands of good studies of neural stem cell biology (the search term "neural stem cell" results in over 6000 references in PubMED. One of the most important aspects of neural stem cells is their choice of cell fate. Many potential therapeutic applications will call for the replacement of specific cell types, such as dopaminergic neurons for Parkinson disease, glutamatergic neurons for stroke, and oligodendrocytes for demyelinating disease or spinal cord injury. One way to manipulate neural stem cells cell fate is through the use of trophic factors added in vitro or in vivo. For example, the addition of a pulse of platelet-derived growth factor to embryonic cortical progenitors promotes neuronal differentiation,[41] whereas ciliary neurotrophic factor and leukemia inhibitory factor promote astrocytic differentiation.[42] A variety of other trophic factors and combinations of factors have been identified that appear to influence neural stem cell fate.

Another way in which cell fate can be influenced is through the manipulation of transcription factor expression. For example, the forced expression of Neurogenin 1 in cultured embryonic telencephalic stem cells induces neuronal differentiation.[43] On the other hand, overexpression of a constitutively activated Notch receptor results in a glial cell fate.[30,44] Transcription factors may also dictate the subtype of neuron that is produced from stem cells. For example, expression of the transcription factor Nurr1 will promote the appearance of dopaminergic neurons in cultures that would otherwise have a low yield of such neurons.[45,46]

In addition to the manipulation of specific transcription factors by overexpression or knockdown, epigenetic regulation of transcription as a means of manipulating cell fate is also under investigation. As is the case with many other cellular systems, DNA methylation plays a key role in the determination of neuronal or glial fate in undifferentiated neural stem cells.[47] Manipulation of methylation status through small molecules may prove to be an effective means of obtaining large numbers of neurons, glia or even uncommitted cells. Another means of epigenetic regulation of neural stem cell fate lies in small RNAs. Micro-RNAs are under intense study as means to regulate the transcription or translation of large numbers of mRNAs. We are, in fact, studying micro-RNA profiles of human neural stem cells. Small RNAs may also play direct roles in transcriptional regulation. For example, Kuwabara et al[48] identified a small RNA that binds to the REST transcriptional machinery to regulate neural stem cell fate.

One aspect of neural stem cell biology that has come under greater scrutiny over recent years is the neural stem cell cycle. We and many other groups have begun to unravel key regulators of neural stem cell proliferation, in the anticipation that manipulation of these pathways will allow for a greater degree of control. Studies have demonstrated that neural stem and progenitor cell proliferation not only ultimately determines the number of cells within the CNS, but that the cell cycle is also closely linked to cell fate (for review see Ohnuma and Harris[49]). In one series of experiments, it was demonstrated that lengthening of the cell cycle caused cortical progenitors to undergo differentiative divisions, whereas

shortening the cell cycle results in more self-renewing divisions.[50] In our own work, we have demonstrated that deletion of *PTEN* recruits more stem cells into the cell cycle, enhances proliferation and maintains neural stem cells in a state competent to produce all 3 lineages, rather than becoming gliogenic over time in culture.[20] Numerous specific genes/pathways have been identified as important regulators of neural stem cell proliferation, many of which are important for several other cell types, including other stem cells. Some of these are: *Bmi-1*,[51] *P21*,[52] *nucleostemin*,[53] *maternal embryonic leucine zipper kinase*,[38] *P53*,[54] *Rb*,[55] and *AKT*[56] among others.

Stem cells by their nature are hearty cells; in order to serve as a reservoir of replacement cells, one would anticipate that they are capable of survival under conditions that other cell types would not. Such appears to be the case with neural stem cells. When we placed neurospheres in a very simple medium, supplemented only with insulin and transferrin, we found that neural stem cells were capable of surviving for prolonged periods (Erickson RI and Kornblum HI, unpublished data, 2003). One mechanism by which neural stem cells may promote their ability to survive is via an ability to exclude toxic compounds through membrane transport pumps. As stated above, neural stem cells express transporters that eliminate fluorescent dyes.[34] These same transporters are responsible for chemotherapy resistance in cancer cells.

Sources of Neural Stem Cells for Therapeutic Use

Mobilization of Endogenous Neural Stem Cells

One potential source of neural stem cells for neural repair is through the mobilization of endogenous stem cells. Numerous studies have described various interventions that enhance or inhibit neurogenesis in the adult mouse brain. As might be expected from in vitro studies, infusion of EGF and bFGF results in the proliferation of cells in the SVZ,[57] although very few of these cells become neurons that survive beyond a brief time. However, in one study, growth factor infusion appeared to enhance replacement of lost hippocampal pyramidal neurons after hippocampal ischemia.[58] Cerebral infarction itself results in the mobilization of SVZ cells.[59–61] It is not clear, however, whether the observed mitotic response is in stem cells themselves or committed progenitors. In the hippocampus dentate gyrus, neurogenesis is enhanced by a variety of stimuli, including seizures,[62] exercise[63] and even antidepressants.[64] Again, it has not been clearly established whether observed effects of these interventions are on stem cells or on a more committed progenitor.

In order to effect neural repair, after proliferation, endogenous stem (or committed progenitors) need to undergo differentiation and migration to appropriate sites. Although fundamental mechanisms of differentiation are highly studied, there is not as much known about the process of migration, especially in the injured or damaged brain. Undoubtedly, a number of factors, including inhibitory and stimulatory molecules will influence this process. Studies with transgenic and knockout mice are shedding some light on endogenous cell migration. In one study, Tsai et al demonstrated that activation of the EPO receptor by erythropoietin is necessary for normal neural progenitor migration to the site of injury after focal ischemia.[65]

Sources of Exogenous Stem Cells

A large number of studies have explored transplantation of neural stem cells and their progeny in a variety of model systems and even in limited clinical investigations. These studies incorporate a wide variety of different sources for neural stem cells, as well as different methods for isolating and culturing them. Very few, if any studies have been done to directly compare these variables, and, until such studies are done, little can be said to support one over the other. Here, we will list some of the potential sources that can be used:

Cultured Fetal Rodent Cells

These cells can be propagated readily for relatively long periods of time. They have the added advantage of allowing for allogeneic transplantation in rodent models of disease or injury. The ease in which these cells can be cultured has allowed for numerous studies of neural stem cells molecular biology, paving the way for preclinical work. Obviously, they have the disadvantage for preclinical work, in that human neural stem cells may theoretically behave differently than rodent cells, and thus preclinical studies with rodent cells would have to be repeated with human cells before clinical trials.

Cultured Adult Rodent Neural Stem Cells

These cells are somewhat more difficult to culture than are fetal cells, but fairly large numbers of cells can be obtained. They are obtained from the striatal SVZ or the hippocampus. One theoretical advantage over fetal cells is that the population is relatively stable in its characteristics and may be more relevant to the stem cells that exist in the adult human brain. However, like fetal rodent stem cells, there is no guarantee that the biology of adult murine neural stem cells will mimic that of human cells.

Fluorescence Activated Cell Sorting–Enriched Rodent Neural Stem Cells

As described above, several protocols exist to enrich (but not purify) neural stem cells using fluorescence activated cell sorting, based on cell size,[33] dye exclusion,[34] antigenic characteristics[36] and promoter-based fluorescent protein expression.[37] The advantage of using sorted cells is that one would theoretically be transplanted a well-characterized population without introducing culture artifacts, such as aneuploidy or altered gene expression. The disadvantages are that the sorting process can dramatically diminish cellular yield, making obtaining sufficient numbers of cells for transplantation a difficult task. To obtain sufficient numbers of cells, one can culture after sorting, but this would then introduce potential cellular heterogeneity and culture artifact.

Enriched or Cultured Fetal Human Neural Stem Cells

Putative fetal neural stem cells can be obtained commercially or from aborted fetuses. One company (StemCells, Inc) uses cells enriched from fetuses by sorting for the CD133 antigen, using a proprietary antibody, followed by culture as neurospheres.[35] The cells are stored in a frozen state and reani-

mated before transplantation. In order to perform preclinical studies, the cells are implanted into immunodeficient rodents. This may not mimic precisely the conditions present in an immunocompetent human transplant recipient. Although the vast majority of the cells express the CD133 antigen after culturing, it is not clear what the true number of neural stem cells within each culture will be. Additionally, when using such cells, one must take into account individual variation from fetus to fetus. Clinical trials are now being initiated to use these cells for enzyme replacement in neuronal ceroid lipofuscinosis (Batten disease; http://www.stemcellsinc.com/clinicaltrials/clinicaltrials.html). This application uses the ability of neural stem cells to serve as "Trojan Horses" by providing enzymes to other other cells within the brain, a concept that has been established in rodent models of enzyme deficiencies.[66]

Embryonic Stem Cell–Derived Neural Stem Cells

Both murine and human embryonic stem cells can be cultured in such a way that they yield a large number of neural stem or progenitor cells.[67–72] These protocols, especially with human embryonic stem cells, have not been fully optimized or tested in a large number of cell lines, but could theoretically yield an extremely large number of cells. One could transplant the cells as neural stem cells, or induce further differentiation, such as down a motor neuron lineage for repair of motor neuron degeneration. All caveats about using cultured cells apply here. Additionally, it is well known that embryonic stem cells themselves will form terratomas on transplantation,[73] so undifferentiated human embryonic stem cells must be excluded from any preparation.

Predifferentiated Progenitors/Precursors

Most potential applications for neural stem cell implantation actually seek to replace differentiated cells, rather than undifferentiated stem cells. Implantations of fetal tissue, in effect, can supply cells that are differentiated or fate-committed, but still possess sufficient plasticity to incorporate into the host CNS. However, there are several drawbacks to using fetal tissue, including cellular heterogeneity, the inability to obtain sufficient numbers of donors, and difficulty in sample preparation. One reasonable strategy for cell replacement would be to expand neural stem cells in vitro and then implant cells after in vitro treatments designed to induce fate commitment and early differentiation steps along desired lines, such as motor neurons or oligodendrocytes.

The list above is in no way the only ways that one might use cellular implantation to replace missing CNS elements. Cells derived from the bone marrow,[74] skin,[75] teeth[76] and heart,[26] for example, have been demonstrated to give rise to neurons or, at least, cells with many of the characteristics of neurons. It is possible, therefore, that tissue other than the CNS may ultimately prove to be as effective and more readily accessible than CNS stem cells.

Potential Applications of Neural Stem Cell Biology to Stroke

The potential uses of neural stem cells for stroke are multiple, but still largely theoretical. Exogenous neural stem cells or neural stem cells that have been predifferentiated to specific neuronal subtypes could potentially be implanted into the injured brain to partially restore neuronal networks. Alternatively, endogenous cells could be mobilized to provide neuronal replacement. In addition to directly replacing lost neurons, it is conceivable that neural stem cells could be used in other ways. For example, implanted neural stem cells or progenitors derived from them could be used to generate myelinating cells or astrocytes to provide support for neurons that remain in the infarcted regions. Implanted cells could also be used to provide therapeutic gene products as well, in order to promote repair. Because neural stem cells normally reside within the CNS, it is reasonable to believe that they will survive and integrate into the host nervous system more readily than non-neural tissue.

Conclusions

Neural stem cells have become one of the most intensively studied cell types in biology. Despite a huge increase in our basic understanding of neural stem cells, a great deal of knowledge needs to be attained before optimally using stem or other cells to achieve functional repair. We need to better understand how to regulate implanted or stimulated cells in the environment of disease and injury, and how to get the cells to become functionally active.

Disclosures

None.

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