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COMMENTARY

Stem Cells: The Promises and Pitfalls

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Stem cells have reached the pinnacle of scientific acceptance, i.e. they are a "hot topic" in the newspapers and on television talk shows. Even the President of the United States recently devoted an address to the nation on a major policy related to this topic. The reason for the interest is clear; stem cells are believed to provide a tool by which new tissues and organs can be made and by which old ones can be repaired. For the central nervous system (CNS) and other organs, e.g. cardiac muscle, this is potentially of crucial importance because cells lost due to damage from injury or disease are not normally replaced. In the brain, the result is permanent neurological or psychiatric signs or symptoms that depend on the area(s) damaged. The hope and the promise is that stem cells and, in particular, neural stem cells will be capable of repairing and/or replacing the neurons lost to trauma, disease or abnormal aging. The promises are, however, accompanied by pitfalls (Nowakowski and Hayes 2000). Here we discuss four pitfalls, three conceptual and one technical, that need to be considered as the literature in this field expands.

PITFALL #1: PROLIFERATION AND "STEMNESS" ARE NOT EQUIVALENT

The first issue that must be addressed, of course, is identity: what are stem cells and what are neural stem cells? Stem cells have been defined as "clonogenic, self-renewing progenitor cells that can generate one or more specialized cell types" (Anderson et al. 2001). This means that they are proliferating cells that can produce one or more different types of progeny AND, importantly, can produce more cells like themselves, a property that is generally referred to as "self-renewal." These special properties of stem cells are in significant contrast to the properties of other progenitor cells, precursor cells, transit amplifying cells and other types of proliferating cells that have been identified by various authors. These other kinds of proliferating cells all are generally said to have limited potential. However, although it is clear that all proliferating cells are not equivalent, the varying potential (or "stemness") of different types of proliferating cells is open for debate (Blau et al. 2001). In the CNS, neural stem cells are generally considered to be proliferating cells that can produce neurons, glia, progenitor cells, and also more neural stem cells, whereas progenitor cells are generally considered to be more limited in their potential and can produce only one cell type, e.g. oligodendrocytes. The lesson from this pitfall is simple: a proliferating cell is not necessarily a stem cell.

PITFALL #2: IN VITRO IS NOT THE SAME AS IN VIVO

Because there are no clear-cut markers for stem cells, the identification of a cell as a stem cell is generally achieved retrospectively through an examination of its progeny. This identification process is more easily achieved in vitro (Anderson et al. 2001) than in vivo because only in vitro can one be sure that one is not only following the progeny of a single cell but also that one accounts for *all* of the progeny. In addition, the in vitro environment is more controlled and provides opportu-

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nities for multiple observations through time of the same cells, while in vivo experiments allow only a single temporal picture of any stem cell and its progeny. However, to reach an understanding of the complex issue of neural stem cells, it is necessary to identify them in vivo and also to define their potential in vivo. In part, this is necessary because of the possibility that the mere act of putting cells in vitro may affect their proliferative (and other) characteristics (Sherr and DePinho 2000); more directly, it is necessary because therapeutic advances will require in vivo manipulations. The difficult questions related to neural stem cells in vivo are tackled by three articles in this issue. All three deal with various aspects of the behavior of stem cells. Taken together, these articles review the field broadly, covering: (1) the role of neural stem cells during the developmental period and also during the complex tissue reorganization that is generally referred to as plasticity (Vaccarino et al. 2001); (2) the intriguing idea that there exist neural stem cells in the neocortex that can be coaxed to become neurons (Magavi and Macklis 2001); (3) the idea that one population of proliferating cells in the adult brain, i.e. those in the dentate gyrus, may be involved in depression and stress and that neurogenesis in the dentate gyrus can be influenced by antidepressant therapies (Duman et al. 2001). Each of these papers reflects the current state of the art of one specific area in this rapidly growing field, and each takes a different approach toward illuminating the roles, properties and potentials of stem cells in vivo.

PITFALL #3: RULES DURING DEVELOPMENT AND RULES IN THE ADULT MAY DIFFER

Vaccarino (Vaccarino et al. 2001) points out that the mechanisms operating to control stem cell proliferation during development are likely to be "re-used" in the adult animal. This is a reasonable working hypothesis, and Vaccarino details some of the molecular controls on this proliferative population. What, exactly, are the cellular behaviors that these molecular controls are controlling? During development, the role of neural stem cells is to build the diverse components of the nervous system. This occurs in an orderly and coordinated fashion, so that the right numbers and classes of cells are produced in a precise sequence. At the earliest stages after the formation of the neural tube, the proliferating cells of the CNS line the ventricles forming a proliferative zone called the ventricular zone. The proliferating cells themselves form a pseudostratified ventricular epithelium (or PVE) that is relatively uniform in its histological appearance regardless of its location in the neuraxis and relatively unchanging as a function of developmental time. From this seemingly uniform population of proliferating cells the diversity of the nervous system develops through differential gene expression that defines segmental (e.g. spinal cord, brain stem, telencephalon), circumferential (e.g. alar vs. basal plate), and radial (e.g. layers and laminae) specializations in form and function. The best studied portion of this extensive proliferative population are those cells that produce the neocortex. For the neocortical PVE we know for example that in mouse from the time of production of the first neuron until the end of neocortical neuronogenesis there are 11 cell cycles during a 6-day period. At the end of this period, the PVE involutes, and the proliferating cells disappear. During development the length of the cell cycle changes, and the proportion of cells that re-enter (P cells) versus leave (Q cells) the cell cycle also changes (Takahashi et al. 1996). This seems to be accomplished by a cycle-by-cycle adjustment of the proportions of the three possible types of cell division, symmetric non-terminal (2 P cells), symmetric terminal (2 Q cells) and asymmetric (1 P cell and 1 Q cell). As a result, the dynamic changes expected and appropriate for a developing organ occur, i.e. during an early expansion phase there are more P cells produced than Q cells, and the brain continues to expand as new neurons are produced, and during a late extinction phase, there are more Q cells produced than P cells as the proliferative population involutes. The result is a brain that grows during the expansion phase and continues to produce neurons until the proliferative population is extinguished. Interestingly, calculations made from measurements of P and Q yield a growth rate for the brain and the production of a number of neurons that agrees quite well with the actual facts (Caviness et al. 1995; Takahashi et al. 2001). As detailed by Vaccarino (Vaccarino et al. 2001), growth factors and other small molecules, including FG2 (Ghosh and Greenberg 1995; Vaccarino et al. 1999a,b; Raballo et al. 2000), PACAP (Nicot and DiCicco-Bloom 2001; Suh et al. 2001), IGF-1 (Drago et al. 1991), NT3 (Ghosh and Greenberg 1995) may all play a role either as a mitogen or an anti-mitogen. In addition, proliferating cells in the ventricular zone are interconnected by gap junctions (Bittman et al. 1997; Bittman and LoTurco 1999) and express GABA(A) receptors, indicating (Owens et al. 1999) that cell-cell signaling (Owens et al. 2000) may also play a role in regulating the dynamic behaviors and Q/P decisions of the proliferating cells of the ventricular zone, although it remains to be determined how these molecules interact with the cell cycle machinery of the proliferating cells (Dyer and Cepko 2001).

The proliferative population of the developing brain may not, however, completely disappear at the end of the developmental period. Two clear examples of continued cell proliferation throughout adulthood have been well documented. The first of these is in the anterior part of the subventricular zone and the rostral migratory stream, where extensive proliferation produces

neurons (and glia) for the olfactory bulb (Luskin 1993; Doetsch et al. 1999). The second well understood example is in the dentate gyrus, where a proliferative zone in the hilus produces neurons and glia (Gage et al. 1998); this second population is the topic of the paper by Duman et al. in this issue (Duman et al. 2001). A third population of stem cells has been suggested to reside in the substance of the brain itself in the form of cells with astrocyte-like properties (Laywell et al. 2000). The existence of this population is part of the analysis of Magavi and Macklis (Magavi and Macklis 2001). How do these adult stem cell populations behave and how are they regulated? Are the rules unique to adult stem cells or, as suggested by Vaccarino (Vaccarino et al. 2001), do the mechanisms from development apply? In one clear way, the adult stem cells are distinctly different from the PVE of the embryo in that they do *not* form a distinct epithelium with interkinetic nuclear movements. They are instead more similar to the proliferating cells of the secondary proliferative population found in the subventricular zone of the embryo. In addition, one might speculate that the adult neural stem cells, rather than following an orderly sequence of events to produce specific products at specific times, respond instead to local environmental influences to produce an output appropriate for the conditions. The experiments of both Duman (Duman et al. 2001) and Magavi and Macklis (Magavi and Macklis 2001) are oriented toward this possibility. However, at this stage we do not know if the various neural stem cell populations that exist in the adult animal have uniform or disparate potential (i.e. Pitfall #1). The best studied of these populations is the one that resides in the dentate gyrus, and that is the topic of the Duman et al. contribution, so let's examine this population a bit more carefully.

In the case of the dentate gyrus, a relatively small number of proliferating cells reside in the hilus. This population is derived from the proliferating cells of the hippocampal PVE during embryonic stages (Nowakowski and Rakic 1981), and in the rodent this population becomes firmly established around the time of birth. During the early postnatal period in rodents, this intrahilar proliferative population produces about 80% of the cells of the granule layer of the dentate gyrus (Bayer and Altman 1975), but these events occur prenatally in monkeys and humans, reversing the proportions to 80% prenatal and 20% in the early postnatal period (Nowakowski and Rakic 1981). Interestingly, during the early postnatal period, proliferation and survival of the output from this population are significantly affected by NMDA-related agonists and antagonists (Gould et al. 1994) suggesting that experience mediated by axonal inputs might affect neuron production during this period. In the adult dentate gyrus, the population of proliferating cells is small compared with the size of the total population of neurons. However, the numbers of proliferating cells and the numbers of neurons they produce varies even within a single species (Boss et al. 1985; Kempermann et al. 1997). In monkey, the number of proliferating cells is about the same as in the rat, but due to the larger size of the monkey dentate gyrus they comprise a much smaller proportion of the total population (Kornack and Rakic 1999). The output from this population consists of neurons, glia, cells with an unknown phenotype (Kempermann et al. 1997, 1998a,b), and cells that die (Hayes and Nowakowski, unpublished observations). The neurons produced in the adult become granule cells (Crespo et al. 1986) and seem to grow mossy fibers (Stanfield and Trice 1988) and to become integrated into the circuitry of the brain (Markakis and Gage 1999). The output is also apparently stable enough to affect an increase in the volume of the dentate gyrus over the course of several months (Bayer 1982). This proliferation seems to persist for the lifetime of the animal, although there is some reduction at later ages (Kempermann et al. 1998a). The paper by Duman et al. (2001) in this issue extends the work of others and shows that the output of this proliferative population, i.e. "neurogenesis", and also the number of BrdU labeled cells can be affected by pharmacological and other manipulations including behavioral experience (Kempermann et al. 1998b). How this proliferation is maintained and variability in output is achieved are unclear because precise measures of the behavior of this proliferative population have not yet been made, although we now know that the cell cycle in rat is about 25 h (Cameron and McKay 2001). The simplest way to imagine this maintenance is to assume that the proliferating population in the adult dentate gyrus is a homogeneous population growing at approximately "steady-state", i.e. with little or no net change in the size of the proliferating population with each pass through the cell cycle. The simplest such proliferating population would consist entirely of cells that divide asymmetrically, i.e. with each pass through the cell cycle one daughter cell re-enters the S-phase and remains a proliferative (or P) cell, and the other daughter cell exits the cell cycle to become a post-mitotic (or Q) cell (Figure 1, left panel), i.e. the Q cells comprise the output (including neurons) from the proliferative population. If the dentate gyrus proliferative population were of this type, then each proliferative cell in the dentate gyrus would have exactly the same behavior, at least with respect to the fates of the two daughters that are produced at each cell cycle. Variable output (such as that demonstrated by Duman et al. 2001) from a population with these properties could only be achieved by affecting the survival of the Q cells. For this type of population, the number of P cells would be invariable because the population can divide only asymmetrically. In Figure 1, panels B–E, an alternative form of steady-state growth is presented. Here the proliferative fates of

the two daughter cells are not correlated (i.e. during early G1, each daughter cell interacts independently with the environment to make its decision to exit the cell cycle or not), and all three types of cell divisions occur. However, because P = Q = 0.5 the mixture of cell divisions at each cell cycle is 1:2:1 and both the size of the proliferative population and the output are constant over the lifespan of the proliferative population. For this alternative form of steady-state growth, variable output (such as that demonstrated by Duman et al. 2001) could be achieved by slight changes in P and Q from the steady-state value of 0.5; this would change the mixture of cell divisions from 1:2:1 and simultaneously affect the size of both the proliferative population and the output from the proliferative population. Thus, the more complex populations schematized on the right side of Figure 1 are possibly more responsive to the sorts of manipulations (e..g, anti-depressants and others as reviewed by Duman et al. 2001) that affect the production of the neurons in the adult dentate gyrus. At this time, there is insufficient data to evaluate whether either of these possible models (or perhaps some other one) reflects the situation in the living animal. Note that, in either case, in order to persist for the lifetime of the animal, the lifespan of the lineages as shown in Figure 1 must correspond to the lifetime of the animal. In addition, in either case, the situation is dramatically different from what happens during development, where the proportions of cell divisions change dramatically as development proceeds (Takahashi et al. 1996), thereby limiting the lifespan of the proliferative population.

PITFALL #4: TECHNICAL LIMITATIONS OF BROMODEOXYURIDINE (BRDU) LABELING

The issue of how this adult proliferative population is regulated is addressed by Duman et al. (Duman et al. 2001). They show that various anti-depressant treatments increase the numbers of cells per dentate gyrus that can be labeled by BrdU. These are interesting and important findings for they indicate that changes in the proliferating population in the dentate gyrus are correlated with depression and possibly also are affected by the drugs used to treat depression. This raises the issue of how these drugs affect the possible lineages that are shown in Figure 1 asking (but not yet answering) what is the cellular effect of these anti-depressants vis-a-vis the proliferative process. In addition, these studies and also those of Magavi and Macklis (in this issue) also point out the dependence of this field on BrdU for assessing "neurogenesis." The dependence on this single method is an important consideration.

BrdU labeling was introduced as a tool for studying cell proliferation in the developing nervous system (Nowakowski et al. 1989); it is unclear how well that tool functions in adult animals. As an analog of thymidine, BrdU is a marker for DNA synthesis and not necessarily a marker for cell proliferation. There are several consequences of this basic fact. First, BrdU labels only cells that are synthesizing DNA; thus, a single injection of BrdU will label cells in the S-phase (Nowakowski et al. 1989), and the S-phase is a small proportion of the whole cell cycle (Nowakowski et al. 1989; Takahashi et al. 1995). It must be considered that relative changes in the length of the S-phase with respect to the total length of the cell cycle could result in the appearance of more labeled cells with a given paradigm. For example, an increase in the length of the S-phase by 25% with no change in either the length of the whole cell cycle or the actual number of proliferating cells will yield an *apparent* increase in the number of BrdU labeled cells by 25%. Thus, although it is clear that the various treatments have some effect on proliferation in the adult dentate gyrus, it is not clear exactly what this effect is. Specifically, it is not determined if these experiments have measured actual changes in the numbers of proliferating cells, or if changes in cell cycle parameters account for the measured differences in

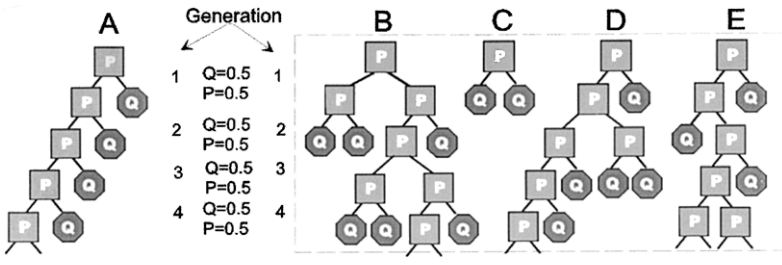

Figure 1. Two populations undergoing steady-state growth. At every cell cycle, both have a constant number of proliferative cells (P) and both produce the same number of post-proliferative cells (Q), i.e. at each generation P = Q = 0.5. In the population on the left, all cells have the same lineage in which each proliferating cell divides asymmetrically. In the population on the right, the lineages would vary. Here an initial population of four cells (B–E) beget daughters and granddaughters that are assigned a P/Q fate randomly. If the overall population is a mixture (1:2:1) of all three possible types of cell division, then the number of proliferating cells and the output of this population are the same as that shown in A at every generation.

the number of labeled cells. Analysis of the cell cycle is necessary to evaluate the results of such experiments and to determine which of several possible interpretations is correct. Second, BrdU can label non-proliferating cells if they are synthesizing DNA. Thus, under some circumstances, for example the massive lesions exploited by Magavi and Macklis (Magavi and Macklis 2001), it is essential to determine if the observed BrdU incorporation is associated with: (1) DNA replication and cell proliferation or (2), with other conditions during which DNA is known to be synthesized in cells, i.e. DNA repair (Selden et al. 1993), apoptosis (Katchanov et al. 2001) or (3) with the development of tetraploidy (Yang et al. 2001). The experiments of Magavi and Macklis (Magavi and Macklis 2001) interpreted BrdU labeling as indication that "new neurons" have been made, but the pathology induced by the lesion could cause DNA synthesis to occur in "old neurons." For example, perhaps in a small percentage of the injured cells DNA repair is successful, and the neurons do not die. This possibility is perhaps reinforced by the recent finding that neurons in areas affected by Alzheimer's disease become tetraploid prior to cell death and remain in this state for an extended period of time (Yang et al. 2001); it is plausible that injury such as that produced by Magavi and Macklis (Magavi and Macklis 2001) could produce a similar phenomenon. One simple criterion for differentiating between replication and other causes of BrdU incorporation is to demonstrate the existence of a appropriate number of BrdU labeled mitotic figures that would appear as the cells labeled in S phase pass through G2 and enter M (Nowakowski and Hayes 2000). Labeled mitotic figures in appropriate numbers would confirm that the increased BrdU incorporation is associated with proliferation and would not appear in a population undergoing DNA repair or with DNA replication in cells becoming tetraploid.

THE FUTURE

The promises of stem cells for CNS repair and treatment of mental illnesses are profound and cause for enthusiasm among all neuroscientists. The enthusiasm, however, should not blind us to the need for prudence and the rigorous use of the scientific method. The pitfalls described above are all experimentally addressable. Thus, they should be used to guide both our conceptual framework and, more importantly, the design of future studies which incorporate the necessary additional experiments. It is essential to explore and eliminate plausible alternative explanations for continued advances in this field to occur.

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