

Reality and immortality—neural stem cells for therapies

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The successful immortalization of neural progenitor cells via the overexpression of telomerase prompts several questions for those intending to develop somatic stem cell therapies for neurological disease.

A paper in this issue by Roy *et al.*¹ provides a very detailed and beautifully executed demonstration that retroviral overexpression of human telomerase reverse transcriptase (hTERT) can immortalize neural progenitors from the human fetal spinal cord. What's more, the authors show these cells can yield, among a number of cell lines, some that appear to be restricted to a neuronal lineage both *in vitro* and *in vivo*. *In vitro*, these cells seem to express some molecules consistent with a ventral spinal neuronal—in some cases motor neuronal—phenotype. The functional relevance of these molecular markers is suggested through *in vitro* studies of calcium influx in response to depolarizing stimuli and of electrophysiological competence. The cells could be passaged without evidence of senescence, karyotypic abnormality or loss of normal growth control. After transplantation into developing rat fetal telencephalon or spinal cord, no neoplasms formed (at least in the small number of animals studied) and neuronal markers persisted *in vivo* with an absence of glial markers. Those attempting to generate neural cell lines from fat, bone marrow, blood and skin should take notice as to the rigor employed in proving these cells were neuronal, a threshold not yet achieved in the 'transdifferentiation' field.

The success of Roy *et al.* in generating immortalized human neuronal cell lines, together with previous reports of engraftable multipotent human neural stem cell lines (propagated by mitogen and/or genetic enhancement)² and human pluripotent embryonic and germ cell lines (which are

'naturally immortalized')^{3,4}, bring to the fore questions regarding the feasibility of clinical translation. These, in turn, invite reflection on a number of even more fundamental issues regarding cellular therapies for neurological diseases (maladies that mirror the challenges in other organ systems as well). Although this article cannot provide definitive answers, it attempts to frame some of the salient questions.

Exogenous versus endogenous cells

The question of whether to implant exogenous progenitor cells or to mobilize a patient's endogenous ones has become pertinent to many organ systems. For the central nervous system (CNS), most studies that have examined the spontaneous behavior of endogenous progenitors in nonneurogenic regions in the intact and injured adult mammalian brain have found neuron replacement to be meager^{5–8}, if present at all⁹, very restricted, short-lived and functionally insignificant. The small number and low survival of incipient neurons might reflect unfavorable microenvironmental conditions for neurogenesis and/or survival, perhaps resulting from a lack of appropriate trophic support, exposure to toxic factors emanating from damaged tissue¹⁰ or simply the absence of appropriate developmental cues.

To overcome these difficulties, several strategies have been proffered to expand the pool of neuron-yielding endogenous progenitors. The success of these techniques in practice remains to be determined. However, even if endogenous stem cells could be recruited, induced to reenter the cell cycle and coaxed to yield relevant neurons, there remains the challenge of generating adequate numbers of proper phenotypes (without creating deformations or tumors) in correct distributions and with sufficient integrative capacity. The most effective therapies will likely entail mobilized endogenous cells supplemented by exogenous cells in particular developmental states. Therefore, transplantation will probably play some role.

From whence the cells?

Given that, in almost every organ system, the progenitor or stem cell population to be abstracted for grafting is small, what is the most efficacious yet safest method for expanding it *ex vivo* such that the cells maintain genetic fidelity from passage to passage without phenotypic drift or senescence? Pluripotent embryonic stem (ES) cells are 'naturally immortalized' and thus provide an unlimited supply of rapidly dividing reagents that can yield theoretically any implantable cell type desired. In dealing with ES cells, however, one must be certain to direct them invariantly down a given lineage and to create safeguards against the appearance of inappropriate nonneural cells, conversion to teratocarcinomas or the emergence of autonomous organs. Although progress is being made in instructing the differentiation of ES cells toward particular neural cell types^{11,12}, knowledge of the relevant developmental mechanisms remains incomplete.

Why not, therefore, use progenitors derived directly from the tissue of interest? Such 'somatic' stem or progenitor cells require less 'taming.' For example, if one starts with a neural progenitor cell, much of the developmental instruction has already taken place; neural progenitor cells have already 'learned' that their 'address' is within the CNS.

But these cells, too, have their limitations. Human somatic stem cells are often slow to expand and, unless genetically augmented, typically senesce. Furthermore, particularly in autograft paradigms, there are theoretical limitations that transcend solely the technical hurdles of isolation, expansion, and differentiation. Although autografts of immunologically compatible and 'ethically neutral' adult stem cells are often touted as being useful for such neurodegenerative diseases as Huntington and amyotrophic lateral sclerosis (ALS), such cells may not be optimal for genetically based diseases—one may be reimplanting adult stem cells that contain the same genetic defect present in the target tissue and with the same susceptibility to degeneration.

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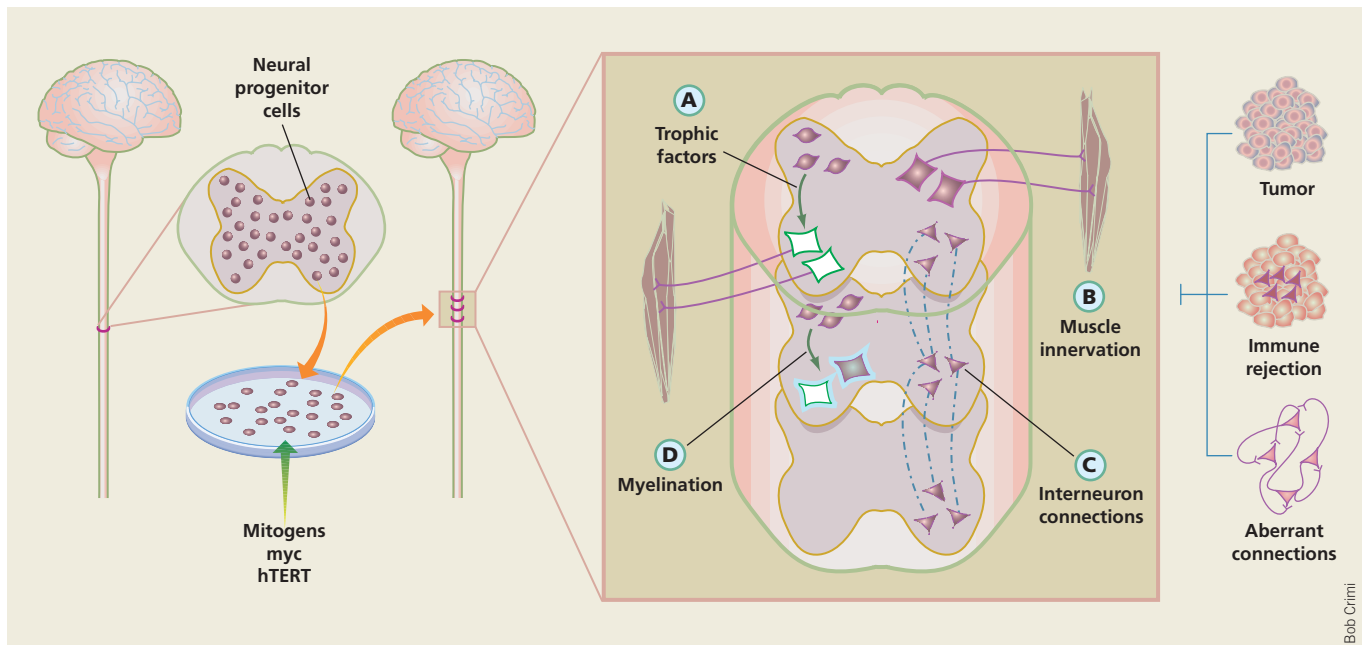


Figure 1 Neural stem and progenitor cells: production, therapy and safety concerns. Left: neural progenitor cells may be abstracted from the CNS region (e.g., spinal cord) and age (e.g., fetal) of interest. Their expansion and proliferation is augmented *in vitro* either by exposure to mitogens or by transduction of cell cycle regulatory genes. Middle: after implantation, the progenitors may act through a number of potential mechanisms, depending on the therapeutic goals. They may become cells (often glia or immature progenitors) that provide molecules for trophic, protective or regenerative support for endangered host cells (A); differentiate into replacement neurons that send projections to host muscle (B); yield interneurons whose projections reconnect local neuronal circuits (C); or become myelinating cells that facilitate the conduction of damaged axons or dendrites. Right: potential adverse outcomes include the generation of disruptive tumors, adverse inflammatory reaction or immune-mediated rejection, and formation of aberrant neural connections affecting healthy tissue. Middle panel: engrafted cells, purple; host neurons, white.

Notwithstanding their limitations in therapies against genetically based diseases, autologous neural progenitor cells still may prove useful for trauma-based or acquired deficits. Here too, however, circumspection is warranted. Grafting a patient's own cells might circumvent ethical and immunological concerns, but the practical hurdles could be daunting. With each new patient, the healthcare team would be confronted with the prospective isolation, expansion, decontamination, characterization and directed differentiation of cellular reagents with its attendant costs in time, resources and personnel, and with potential variability between preparations, patients and institutions. If one is to contemplate using non-neural adult stem cells for neural purposes, a low-efficiency transdifferentiation event (if it exists at all) must be scaled up to a clinically relevant level, which presupposes a knowledge of the signals involved and an ability to provide them in a controlled way—a goal far from realized. It is becoming increasingly recognized that the optimal time for dealing with CNS injury using stem cells is during the acute or subacute period. It is possible that, by the time adequately characterized cells are optimized, the win-

dow of opportunity for using them may have passed.

An alternative to autografts is the use of established, somatic stem or progenitor cell lines that might serve as 'universal donor cells.' These have the appeal of being homogeneous, stable off-the-shelf reagents, well characterized and maintained under good manufacturing practices, readily available in limitless quantities for the acute phases of an injury or disease and documented to be safe. Their downside, of course, is the possibility of immune incompatibility (which might be addressed through additional engineering or immunosuppressive agents) and the fact that the best source for such universal lines may be the embryo or the fetus, where various immunogenic markers are less prevalent, rather than the adult. Another concern that must be addressed whenever any expanded or passaged cell—whether propagated by chronic exposure to mitogens or by genetic enhancement²—is placed in the body is the risk of producing neoplasms.

The present paper by Roy *et al.* helps debunk the myth that gene-based methods are inherently dangerous and must be avoided *a priori*. Indeed, there is speculation that unabated exposure to mitogens, such as

epidermal growth factor or basic fibroblast growth factor, could promote mutations by selecting for particularly avaricious receptors and catalyzing de-differentiation. But the expression of certain transgenes—for example, the gene encoding hTERT, which is not a foreign gene—may be modulated by the cell's intrinsic regulatory mechanisms or may blunt senescence and stabilize the phenotype. It is wrong, the data in this paper argue, simply to dismiss such lines as 'transformed'—a term that, although often used casually and pejoratively to describe immortalized lines, is in fact erroneous in this context because it has a very precise definition in the oncobiological literature that is inappropriate to most stem or progenitor cell lines. Indeed, some of the criteria for neoplastic transformation, which include lack of contact inhibition, growth in soft agar, tumorigenesis in nude mice, loss of growth control, anaplasia and karyotypic instability, have been specifically ruled out by these authors. The gene encoding hTERT is, by the 1980s definition, an oncogene (that is, associated with neoplasms), as are many other genes, such as *wnt*, *bcl-2*, *trk*, *erbB* and *myc*, that are now understood to play normal developmental roles and would no longer be classified as

oncogenic (though their association with neoplasms now seems explainable). Roy *et al.* have quite nicely shown that the simple over-expression of hTERT is likely safe and effective in generating reagents of potential scientific and therapeutic utility.

How plastic should transplanted cells be?

The ability to generate neuron-restricted lines raises questions regarding what it might take to repair a dysfunctional CNS and what type of cell might require replacement. Before transplantation, what is the optimal degree of differentiation of a neural progenitor cell for a particular disease: a predifferentiated, rigidly committed state or a less differentiated and more plastic state, which would allow the cell to mature *in situ*?

The complexity of the above question is compounded by three emerging realizations. First, for many diseases, reconstruction of the damaged milieu may require replacement of multiple cell types and multiple proteins—the cells that have died are not only neurons, but also support cells that detoxify the environment, myelinate the axons and dendrites, supply ongoing trophic and matrix support and provide reservoirs for ongoing cell replenishment. Second, the interaction between transplanted neural progenitor cells and the recipient host is a dynamic reciprocal interaction in which both entities influence one another. The neural progenitor cell inherently expresses genes that are capable of signaling, instructing, remodeling and protecting the host CNS, suggesting another mechanism by which therapeutic outcomes might be achieved: an inherent capacity of neural progenitor cells (without genetic engineering) to create host environments sufficiently rich in trophic and/or neuroprotective support to rescue endogenous cells^{13–21}. Third, for many diseases, we might be unduly presumptuous in assuming that we know which cell type is needed for replacement and what is required to reconstitute a given region and restore function.

As fundamental as these questions are, the answers are quite elusive in the majority of neurological diseases. Ostensibly, a motor-neuron degenerative disease like ALS would seem to be an ideal therapeutic target for the cell lines described by Roy *et al.* Yet unexpected recent findings have forced us to be more circumspect: the death of motor neurons in ALS appears to be intrinsically linked to dysfunction of nonneuronal cells (perhaps astrocytes), suggesting that one might desire a multipotent line that can yield both replacement glia as well as replacement neu-

rons, or perhaps, if a lineage-restricted line is chosen, glial-restricted cells^{22–26}.

The use of neuronally restricted lines in traumatic spinal cord injury presents another interesting discussion. Spinal cord injury is classically viewed as a disease of axons and oligodendrocytes, not neurons *per se*, suggesting that glia would be required rather than motor neurons. Nevertheless, neuronally restricted cells might create bridges via the interspersed of new interneurons throughout a lesion. Still, glial as well as neuronal precursors would seem optimal. Although implanting cells precommitted *ex vivo* to yield a uniform mature neural cell type certainly maximizes numbers (and, in the case of embryonic stem cells, safety), the ability of such cells to accommodate varying environmental cues and to provide other needed cells in an appropriate ratio within the same structure may be compromised.

How rigid is lineage- and region-restriction?

How rigid is 'restriction' of a cell line? The question remains open. Can such a line—over time or if placed in a variety of differentiating conditions—be prompted to circumvent its restriction? The group responsible for the present paper also published a fascinating report²⁷ in which ostensibly human oligodendrocyte-restricted lines did not remain lineage-restricted. In fact, multipotency, including the ability to yield neurons, spontaneously emerged.

Similarly, how truly regionally restricted is a mitotic neural progenitor cell when removed from its context, propagated in a dish and then reimplanted elsewhere? Some of this discussion might be addressed by understanding whether, after transplantation, it is the immature proliferative cells that engraft and then mature into neurons *in vivo*, presumably responding to extant environmental cues², or the post-mitotic, presumably neuronally committed, cells that engraft. Having matured in a dish, the post-mitotic cells may retain signals from their site of origin. It is interesting that the hTERT lines described by Roy *et al.* formed ectopic neurons in the white matter, indicating that they were committed to a neuronal fate, even as mitotic precursors, and could or did not respond to environmental cues. If so, does this suggest that a line must be made from each desired CNS region because restriction to, for example, a cortical neuron, would preclude a line yielding a spinal anterior horn cell? Would we, in fact, prefer to have more plastic lines that are more responsive to envi-

ronmental cues? Might we simply have to make such decisions on a disease-to-disease basis? Or might we need to do co-grafts for very complex diseases involving multiple cell types and multiple regions in order to effect optimal functional reconstitution?

Conclusions

To fully realize the therapeutic potential of neuronal progenitor cells, clinicians and neuroscientists face the following challenges: how to direct such cells (whether endogenous or exogenous) to different CNS regions to yield cells of the right type(s) and number, in the right ratio, in the right location, making the right connections with the right partners without making any wrong connections, and to shield nontargeted cells and regions from such influences. Combinations of cells may be required—various types at perhaps different developmental or differentiation stages for different phases of a given disease (Fig. 1). If so, the answer to the question, "Which cell for which disease?", might in fact vary from disease to disease and structure to structure, to be determined empirically over the next decade.

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