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In situ stem cell therapy: novel targets, familiar challenges

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Tissue engineering approaches for expanding, differentiating and engrafting embryonic or adult stem cells have significant potential for tissue repair but harnessing endogenous stem cell populations offers numerous advantages over these approaches. There has been rapid basic biological progress in the identification of stem cell niches throughout the body and the molecular factors that regulate their function. These niches represent novel therapeutic targets and efforts to use them involve the familiar challenges of delivering molecular medicines *in vivo*. Here we review recent progress in the use of genes, proteins and small molecules for *in situ* stem cell control and manipulation, with a focus on using stem cells of the central nervous system for neuroregeneration.

Endogenous stem cells: the goal of control

Stem cell research has escalated significantly in recent years, with a focus on increased understanding of natural roles of stem cells in cell and developmental biology as well as their potential use for biomedical applications. Embryonic stem (ES) cells have significant potential for tissue regeneration, and readers are referred to recent reviews in this area [1,2]. In addition, tissue engineering approaches involving the *ex vivo* expansion, controlled differentiation (potentially with an appropriate scaffold) and engraftment of either embryonic or adult stem cells (see Glossary) also have significant potential and have recently been discussed [1,3]. However, this review focuses instead on a worthy long-term goal of the stem cell biotechnology field: a regenerative medicine approach for tissue repair focused on the direct manipulation of endogenous adult stem cell pools, which have been found to exist in numerous tissues. Furthermore, although several studies from other tissues will be discussed, we focus on examples from the nervous system that have implications for numerous devastating neurodegenerative disorders.

Direct adult stem cell manipulation offers several advantages. First, harnessing endogenous stem cells circumvents the immunocompatibility issues that accompany the use of embryonic stem cells and allogenic adult stem cells [4]. Second, *in situ* manipulation involves the use of molecular medicines that are more established or 'conventional', including small molecule, protein and (potentially) gene therapies. Finally, these medicines are potentially more economically feasible than *ex vivo* approaches. Although it is clearly more challenging to gain the control over a stem cell's environment to precisely manipulate its function *in vivo*, there has been significant progress in reprogramming endogenous pools, to study both their basic function as well as their therapeutic potential.

Neurodegenerative disorders and adult neural stem cells

Several neurodegenerative disorders have enormous personal and economic costs but have limited treatment options, in part because they often involve severe depletion of neurons by the time the condition is diagnosed. For example, in Parkinson's disease, which results in severe declines in motor function and afflicts >1 million people in the U.S.A. alone, 80% of striatal dopaminergic neurons are lost by the time clinical symptoms are apparent [5]. In addition, amyotrophic lateral sclerosis (Lou Gehrig's disease) affects 2–3 of every 100 000 people and it progressively kills upper and lower motor neurons of the spinal cord and brain resulting in death typically within 5 years [6]. Furthermore, in advanced Alzheimer's disease up to 80% of cholinergic inputs into some regions, such as the temporal lobes of the brain, can be lost [7]. Therefore, for these and other disorders, cell-replacement therapies should be explored for halting or even reversing disease progression.

Over the past decade it has become increasingly clear that the adult central nervous system harbors numerous neural stem cell niches – findings that contradicted

Glossary

Adeno-associated virus (AAV): A 4.7 kilobase DNA virus that can be converted into a vector capable of mediating high efficiency gene delivery to postmitotic cells, such as neurons, resulting in highly sustained gene expression. AAV delivery of genes encoding secreted factors is one effective approach to modulate adult neural stem cell function.
Adult stem cells: An undifferentiated population of cells that retain the ability to proliferate throughout postnatal life and to differentiate into specialized cells to replace those that die or are lost.
Astrocytes: A major cell type of the human nervous system. Although they have been traditionally viewed as cells that only support neurons, recently they have increasingly been found to be involved in modulation of neural signaling through bi-directional communication with neurons.
Dentate gyrus (DG): One region of the hippocampus that harbors active adult neural stem cells.
Hippocampus: A region of the brain involved in processing and transferring information into memory. It is severely affected by Alzheimer's disease.
Neurogenesis: The generation of novel neurons.
Oligodendrocytes: These cells ensheath neurons with myelin, an insulating material that enhances the speed of neuronal signaling (i.e. action potential conduction along a neuron).
Subventricular zone (SVZ): A second region of the adult CNS that harbors neural stem cells active in the adult.

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traditional views that neurons are irreplaceable. Two regions of the brain, the dentate gyrus (DG) region of the hippocampus and the subventricular zone (SVZ) of the forebrain, contain active stem cells that continually divide and generate large numbers of new neurons (i.e. adult neurogenesis) daily throughout human life as reviewed in [8–13]. *In vivo* and *in vitro* studies show that these stem cells can differentiate into neurons, astrocytes and oligodendrocytes (Figure 1), the three major cell lineages of the central nervous system (CNS). Moreover, new neurons can functionally integrate into these regions [14,15] raising the possibility that new cells could functionally repair tissue in a disease model [16]. Furthermore, although active neurogenesis appears to be limited to these two regions, the adult CNS contains quiescent stem cell pools (including within the spinal cord [17,18], substantia nigra [19], optic nerve [20] and hypothalamus [21]) that can be isolated and expanded in culture. Several studies indicate that adult hippocampal and SVZ neurogenesis appears to be involved in aspects of learning and memory, as reviewed in [9,22]; however, the functions of quiescent pools are not understood. Some results indicate these stem cells in the cortex might be involved in regeneration from injury [23–25] although cortical neurogenesis is an area of debate [26,27].

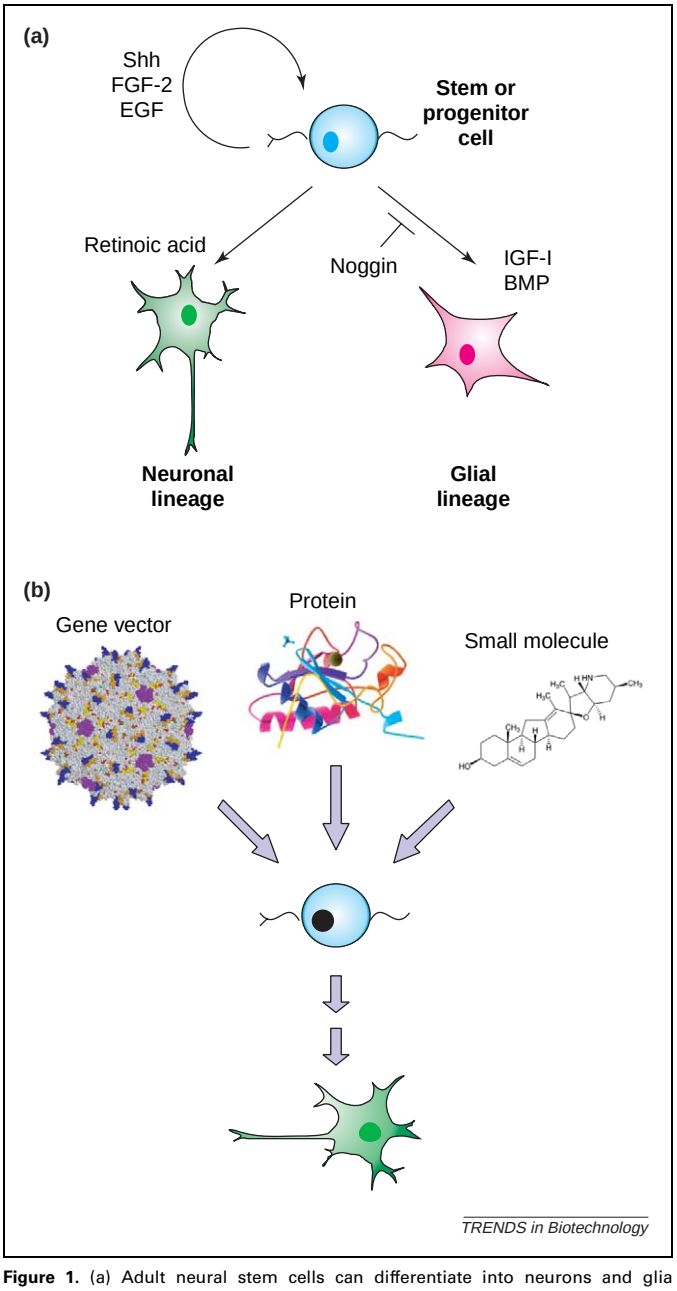

Figure 1. (a) Adult neural stem cells can differentiate into neurons and glia (astrocytes and oligodendrocytes), the three major lineages of the adult nervous system. The instructive signals that have been shown to exert direct effects on adult neural stem cells and that are discussed in this review are shown. These include epidermal growth factor (EGF), fibroblast growth factor (FGF-2), insulin-like growth factor (IGF-I) and bone morphogenetic proteins (BMP). (b) There are three molecular medicines the delivery of which can induce signaling to modulate and control stem cell proliferation and differentiation *in situ*: small molecules, proteins, and genes.

Basic efforts will continue to yield a deeper understanding of the basic biological importance and regulatory mechanisms of these cells. Regardless of the normal roles they have in the adult CNS, neural stem cell niches are highly promising resources that can potentially be harnessed to regenerate tissue. To do so, approaches must be developed to control the proliferation, differentiation and functional integration of these cells, and the quantitative analysis of stem cell function might aid in the design of control strategies for therapeutic applications [28,29]. In addition, disorders arising from inherited genetic mutations will presumably affect differentiated cells that arise from endogenous adult stem cells, requiring the genetic correction or protection of the new cells. Although the stem cell targets of such therapeutic approaches might be novel, the therapies will probably involve the familiar molecular therapeutics of the drug development and biotechnology fields: genes, proteins and small molecules (Table 1). Therefore, the traditional challenges of development and delivery, including efforts to translate results from animal models to human subjects, will still apply.

Gene therapy approaches

Alhough gene therapy has not yet reached the status of a standard clinical approach, gene delivery studies have provided compelling evidence that adult neurogenesis is controlled and modulated by exogenous signals – although this work had the primary goal of identifying new signals that regulate stem cell function. We recently demonstrated that the factor Sonic Hedgehog (Shh), well known for its crucial role in neural development, regulates the proliferation of stem cells in the hippocampus [30]. Specifically, adeno-associated viral (AAV) vector delivery of cDNA encoding Shh to the adult rat brain tripled the number of proliferating cells, which later yielded a threefold higher number of mature neurons. Although stem cell proliferation was stimulated, we relied upon endogenous signals of the neurogenic hippocampus to regulate their differentiation into neurons, and future work might involve more actively inducing this fate. In addition, in the undamaged brain, the survival of immature and differentiated cells is probably tightly regulated, another factor that might require control for tissue regeneration. This work has therapeutic relevance because the hippocampus is a region severely afflicted by Alzheimer's disease. A subsequent study showed that adeno-associated viral vector delivery of cDNA encoding insulin-like growth

Table 1. Comparison of different modes of delivery for stem cell therapy

	Gene therapy	Proteins	Small molecules
Advantages	Ability to directly reprogram stem cells *in situ* through integration and expression of certain genes.	Direct and transient delivery of the therapeutic protein to the stem cell population. More control over the course of administration.	High-throughput screening easily applied to identify groups of synthetic small molecules capable of modulating stem cell behavior as desired. Can be made to be highly specialized for targeting. High purity achieved through synthetic chemistry. Many small molecules are capable of passing through the blood–brain barrier.
Disadvantages	Highly dependent on development of safe and efficient delivery vectors that can also cross the blood–brain barrier. Precise control of gene expression might be a challenge.	Blood–brain barrier might prevent systemic administration of certain proteins, which might require more invasive techniques. Even if systemic administration is possible, there might be adverse side effects in other parts of the body	Adverse side effects of systemic delivery apply again.

factor (IGF-I) to the hippocampus led to a three-fold increase in the number of oligodendrocytes [31]. The finding that IGF-I promotes the differentiation of hippocampal stem cells into oligodendrocytes is relevant to demyelinating disorders such as multiple sclerosis.

In a study of the other neurogenic region of the brain, the SVZ, it was found that bone morphogenetic proteins (BMPs) promoted the astrocytic differentiation of neural stem cells [32,33]. Consequently, adenoviral vector delivery of the BMP antagonist Noggin supported the neuronal differentiation of SVZ stem cells implanted into the striatum [34], a region severely affected by Parkinson's disease. However, in rodents endogenous SVZ stem cells ordinarily differentiate and migrate to supply new neurons to a somewhat distant region, the olfactory bulb. In a recent important study, Goldman and colleagues used adenoviral vector overexpression of Noggin and brain-derived neurotrophic factor (BDNF) to simultaneously suppress astrocytic differentiation and promote neuronal differentiation of endogenous SVZ stem cells. They thereby recruited them to develop into neurons in the striatum, an otherwise non-neurogenic region of the brain [35].

Together, these studies show that gene delivery can efficiently reprogram neural stem cells *in situ*. However, clinical development of a gene therapy approach will depend on challenges in the development of safer and higher efficiency vectors being overcome [36] as well as analysis and validation that the results of these animal studies apply to human neural stem cells because species-specific differences might exist. In addition, for gene delivery vehicles such as AAV that provide long-term gene expression, it might be necessary to implement small molecule gene regulation systems to control expression. Furthermore, each of these studies involved direct injection of vector into the brain, but the development of 'smart' gene delivery vehicles capable of passing through the blood–brain barrier will make combining gene and neural stem cell therapy approaches even more feasible in the future [37].

Protein delivery

Because of delivery challenges and pharmacokinetic considerations, the use of proteins to control stem and progenitor cell function *in vivo* might sound difficult, if it had not already been standard clinical practice for 15 years. Erythropoietin (Epo), granulocyte colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GM-CSF), and other factors have been extensively used to modulate hematopoietic progenitor differentiation in the clinical treatment of cancer and anemia [38]. Although this practice does establish an important precedent, protein delivery to tissues other than blood poses additional challenges, particularly for the CNS in which the blood–brain barrier poses a formidable hurdle. However, there are several situations in which systemic growth factor injection can modulate neurogenesis in the brain. Long term peripheral infusion of insulin-like growth factor I (IGF-I) led to a $>40\%$ increase in the number of proliferating cells and $>70\%$ increase the number of newborn neurons in the adult rat hippocampus [39]. In light of more recent work indicating that direct delivery of IGF-I to the hippocampus induced oligodendrocytic differentiation [31], however, it is not clear whether the effects that peripherally infused IGF-I exerts on hippocampal stem cells are direct or indirect. In another study in which peripheral protein delivery modulated CNS stem cell function, intraperitoneal injection of erythropoietin – previously mentioned for its modulation of hematopoietic progenitor function [38] – nearly doubled cell proliferation and neuronal differentiation in the SVZ [40]. In parallel, Epo significantly improved functional recovery in a middle cerebral artery occlusion stroke model. Together, these studies indicate that systemic protein delivery can modulate CNS stem cell function.

In addition, there are several studies in which controlled, local protein delivery systems were used to modulate CNS function. Osmotic pump infusion of fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) to the lateral ventricles significantly increased cell

proliferation in the SVZ but had minimal effects in the DG of the hippocampus [41]. By contrast, Nakatomi *et al.* found that osmotic pump infusion of FGF-2 and EGF into the lateral ventricles following an ischemic injury induced 4.2- and 9.2-fold increases in the levels of newborn neurons in the CA1 region of the hippocampus, a region where neurogenesis does not normally occur [42]. The newly generated neurons functionally integrated into the neural circuitry and animals treated with the growth factors after injury exhibited considerable functional recovery. The fact that normally dormant populations of stem cells can be stimulated to proliferate and produce functional neurons after injury, potentially through synergy with endogenously released factors, has important implications for the treatment of numerous neurodegenerative diseases. Finally, polymeric controlled release systems have been developed for protein delivery in the CNS [43,44]. In one study, polymeric microparticles carrying nerve growth factor (NGF) enhanced the survival and potentially the differentiation of fetal rat brain tissue co-injected with the particles, a result with potential implications for the treatment of Alzheimer's disease [43]. Such controlled release approaches could be extended to manipulate endogenous stem cell populations with protein factors that cannot exert their effects, or have side effects, when injected systemically.

Small molecules

Small molecules have been a staple of the drug development field and modern approaches of combinatorial chemistry and high-throughput screening have recently been applied to the problem of stem cell control. This promising work has shown that *in vitro*, and in some cases *in vivo*, small molecules have the potential to control numerous aspects of adult stem cell function, including proliferation, differentiation and functional integration. Using gene delivery we demonstrated that activation of the Sonic Hedgehog signaling pathway promoted adult neural stem cell proliferation [30]. Several small molecule agonists of this signaling pathway have recently been generated and tested for bioactivity in numerous assays *in vitro* [45,46] and a recent study employed these agonists to demonstrate that Shh signaling promotes the expansion of telencephalic neural progenitor cells in the postnatal brain [47]. In addition, several small molecule antagonists of this pathway have also been identified, including both natural and synthetic molecules [45,48–50], which might have important implications for treatment of cancers caused by various oncogenic mutations in this signaling pathway. However, gene delivery or other local drug-delivery systems might be necessary to avoid side effects likely to be inherent in systemic delivery of such small molecules in general [51].

Several other compounds have been shown to promote adult neural stem cell proliferation *in vivo*. Lithium, a standard treatment for mood disorders, was shown to modestly promote hippocampal stem cell proliferation *in vivo* [52], and subsequent work indicates that it might actually bias the expanded cells towards neuronal differentiation [53]. In addition to lithium, valproate, another mood-stabilizing drug, has also been shown to increase levels of neuroprotective bcl-2 in the frontal cortex [54] and stimulate the extracellular signal-regulated kinase (ERK) pathway in the rat hippocampus and frontal cortex [55], which is believed to be important for neuronal survival, regeneration, differentiation and structural and functional plasticity. This link between mood-stabilizing compounds and adult neurogenesis suggests a link between neurogenesis and depression [9]. Finally, chronic (14 days) administration of rolipram, an inhibitor of phosphodiesterase type IV (PDE4) that elevates cAMP levels, resulted in a 37% increase in neurogenesis in the dentate DG of the hippocampus of adult mice. These rolipram-induced cells appeared similar to older cells in their morphology, location and differentiation characteristics [56], suggesting that the activation of the cAMP-CREB signaling cascade can be used to increase the available pool of stem cell population in the adult brain.

Small molecule modulators of differentiation have also been identified. Schultz and colleagues have also used a 'chemical genetics' approach in generating and screening large libraries of compounds ($>50\,000$) for agents that modulate stem cell function [57–60]. They first identified molecules that induced osteogenic differentiation of mesenchymal stem cells [58]. In subsequent work, they applied this approach to ES cells and identified compounds that induced the neuronal differentiation of embryonic carcinoma cells and cardiac myocyte differentiation of murine ES cells [57,59]. Not only can this approach lead to the identification of signaling pathways involved in stem cell differentiation, but these compounds also have application for *in vitro* tissue engineering approaches. In addition, future animal studies will reveal whether the work can be extended to *in situ* stem cell regulation in adults.

Finally, combined therapeutic approaches involving small molecules might promote the functional integration of neurons. It is known that myelin in the spinal cord has numerous elements that inhibit the regrowth of neuronal axons after injury and these inhibitors would have to be overcome for stem cells to successfully and functionally repopulate this region. Lu *et al.* combined cAMP treament, previously shown to enhance axonal sprouting after a lesion [61,62], with neurotrophin-3 protein therapy and engraftment of bone marrow stromal support cells, to significantly promote axonal regrowth after spinal cord injury [63]. This work showed that lesioned neurons might be induced to regenerate, and one long-term goal could be to investigate whether such approaches can be further developed and applied to spinal cord stem cells [17,18] to promote the generation and functional integration of new neurons for treatment of amyotrophic lateral sclerosis or spinal injury. These studies show that small molecules have made significant and recent strides in the stem cell control fields, and additional animal studies will indicate whether local delivery systems might be necessary.

Conclusion

The endogenous regenerative capacity of many tissues, particularly the CNS, is limited and new approaches must be developed to enhance these efforts. Embryonic stem cell and stem cell-based tissue engineering research are efforts

that are worthwhile pursuing. However, the stem cell niches that are present in adults are natural 'gifts' that offer potential for tissue regeneration if we can learn how to control them. These cellular targets, many of which have emerged in the past several years, are highly novel; however, the molecular medicines that must be developed to exploit them will fall into traditional categories. Therefore, as challenges in the gene, protein and small molecule therapeutics fields are progressively overcome, their capabilities can be merged with increasing knowledge in the adult stem cell field to develop therapies for diseases that currently have few options, particularly neurodegenerative diseases.

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