NEURAL STEM CELLS: BIOPROCESS ENGINEERING

Arindom Sen, Michael S. Kallos, and Leo A. Behie
Pharmaceutical Production Research Facility (PPRF), Schulich School of Engineering, University of Calgary, Alberta, Canada

INTRODUCTION

Tissue and organ failure account for half of the total annual health care expenditure in the United States (1). The best solution currently available for organ failure is transplantation. However, the demand for organs far outweighs the supply, thereby creating a situation where many people are placed on waiting lists, and will die before receiving a transplant. Moreover, those who receive transplants are typically faced with a lifetime of complications associated with immunosuppression. For incidents of tissue failure (e.g. Parkinson’s disease (PD), diabetes, and arthritis), the primary mode of treatment is the administration of pharmaceuticals. However, pharmaceuticals are typically used to mute symptoms only, and do not address the root cause of a disease, and, thus, rarely halt disease progression.

Regenerative medicine is a rapidly emerging field that may present new potential treatment alternatives for individuals suffering from tissue and organ failure. This interdisciplinary field combines the principles of engineering and life sciences to generate tissues and organs, which can act as biological substitutes that restore, maintain, or improve biological function. This revolutionary new approach to medicine is receiving great interest from both academic and industrial sources, and the occupation of a “tissue engineer” has been hailed as one of the hottest jobs of the twenty-first century. Tissue engineers typically combine scaffolds, cells, and signals to regenerate entire tissues and organs. Unfortunately, mature functional cells do not readily proliferate in culture, and as such are not ideal for tissue engineering applications. However, owing to their capacity to proliferate and regenerate the multitude of cell types that comprise various tissues and organs, stem cells are rapidly emerging as a promising source of autologous or allogeneic cells that may have utility in tissue engineering applications.

Stem cells can be derived from the early embryo (embryonic stem cells (ESCs)) and fetal and adult tissues (somatic stem cells (SSCs)). Their ability to be grown outside the body, coupled with their capacity to divide and specialize into many other cell types has fueled an incredible increase in the amount of research being conducted on the biology, function, and potential clinical uses of these rare cells. Engineers play a key role in linking the basic science of discovery with the final implementation of stem cell technology. This article will focus on the development of bioprocesses for the production of neural stem cells (NSCs) with clinical applications in the treatment of PD and other neurodegenerative disorders. We will show that our initial work using the murine system has provided a good bioengineering starting point, which then accelerated our studies with human cells. The information presented has been applied in our laboratory toward the development of bioprocesses for other stem cell systems, including ESCs (2,3), pancreatic progenitors (4,5), and mammary epithelial stem cells and cancer stem cells (6–8).

BACKGROUND

Stem Cells

Stem cells are primitive cells that have several key hallmarks that set them apart from other mammalian cells, including (i) their ability to proliferate and form copies of themselves (self-renewal) and (ii) their ability to specialize or differentiate into many other cell types (potency).

When a stem cell divides, it can form daughter cells that are also stem cells (self-renewal) or cells that have started to differentiate. Once a stem cell initiates differentiation, it is said to be “committed,” and is called a progenitor cell. Progenitor cells and stem cells are collectively called precursor cells. Progenitor cells do not have the ability to self-renew, although they do have the ability to proliferate. The potency of a stem cell can be defined as (i) totipotent (the ability to form all cells in the body including the extraembryonic tissue—the yolk sac is the only truly totipotent cell), (ii) pluripotent (the ability to form all cells in the body—this includes ESCs), and (iii) multipotent (the ability to form all cell types from a particular tissue—this includes SSCs such as NSCs). SSCs are also referred to as adult stem cells.

ESCs are pluripotent cells derived from the inner cell mass of the blastocyst (9) (Fig. 1a). Their pluripotency has been exploited to investigate the cellular factors needed for in vitro differentiation into a variety of tissue types [reviewed in Ref. 10]. With the recent generation of human ESCs, there is great anticipation for how stem cells might be used in the emerging field of regenerative medicine [reviewed in Ref. 11]. In vivo, ESCs divide and differentiate to form the fetus. In vitro, they have been shown to possess the ability to form any tissue type from all three germ layers (12,13), including those that do not normally regenerate. Due to the ethical and moral dilemmas in using human embryonic stem cells (hESCs), mouse model systems have been developed (14).

SSCs are multipotent and can be isolated from many different tissues in the body including the brain (NSCs), bone marrow (hematopoietic stem cells), and skin...
Neural Stem Cells

NSCs are multipotent stem cells that can be isolated from the mammalian central nervous system (CNS) (16,17). The CNS includes the brain and spinal cord. NSCs have the ability to form all the major phenotypes of terminally differentiated cells found in the CNS, including neurons, astrocytes, and oligodendrocytes (Fig. 1b). Neural precursors (stem cells and differentiated progenitor cells) are usually identified by their ability to proliferate to form colonies in static adherent culture or spherical aggregates (neurospheres) in nonadherent and suspension culture. When these colonies or aggregates are dissociated and replated in fresh medium repeatedly, only NSCs will retain the ability to form new colonies and aggregates (17). Progenitor cells have limited proliferation ability, which means they will not be able to form colonies/aggregates in long-term culture.

Stem cell cultures are by their nature heterogeneous, being composed of a mixture of stem cells, progenitor cells, and more differentiated cells. Complete cessation of spontaneous differentiation or growing pure stem cell cultures has not been demonstrated. Any bioprocess for the expansion of NSCs requires the multiplication of cells (self-renewal) and the subsequent generation of secondary clones from the primary aggregate (17). The frequency of primary aggregates—forming cells will typically range from 10% to 20%, but only a small fraction of those primary aggregates will contain cells that can go on to form secondary aggregates. This means that the frequency of true stem cells will typically be on the order of 0.1% of a culture, depending on the cell line, media, and other in vitro conditions. There are kits available from companies such as StemCell Technologies (Vancouver, Canada) to measure NSC frequency. Multipotency is measured by taking clonally derived aggregates, plating them in adherent culture, and exposing them to differentiating factors—usually serum—for a defined period of time. The resulting colonies can be examined immunocytochemically for markers of neurons, astrocytes, and oligodendrocytes.

Neural Stem Cell Sources

When designing a bioprocess for stem cell production, it is important to consider the source of the cells, as NSCs isolated from different sources will behave differently in culture and will therefore require different process parameters (Fig. 2). NSCs can be isolated from the fetal CNS (16,17), the adult CNS (18), and can be generated from E2Cs (19) and other non-CNS tissues (e.g. bone marrow) (20). Each source has both advantages and disadvantages from a bioprocess point of view. Fetal sources of NSCs offer the advantage of obtaining tissue well in advance of any transplantation, which will allow for cell purification, characterization, and cell banking. Purification is necessary to remove any unwanted cell types such as vasculature and immune cells that may cause unwanted immune reactions in clinical recipients. Characterization of cell numbers, viability, and cell population distribution as well as the precise genomic and proteomic makeup of the cell population will also be required. Cell banking will allow testing to be carried out on the same population of cells that will eventually be transplanted, and will give an "off-the-shelf" product for transplantation in addition to allowing future testing of cells should new concerns arise. E2Cs also offer the advantage of being able to obtain the cell source in advance of any final use, which will allow for cell purification, characterization, and cell banking.
The physical manifestation of this mutation is near the telomere of the short arm of chromosome 4 the IT15 gene (codes for the protein huntingtin) located caused by repeats of the trinucleotide sequence CAG in ment). HD is an autosomal dominant genetic disorder (involuntary, uncontrollable, and often excessive move- DA neurons and development of disabling dyskinesias usually limited to 3–5 years due to continued loss of relieves some of the symptoms of PD, its benefits are drug development and testing, and biological potential uses of neural stem cells potential uses of neural stem cells the need for large-scale stem cell production bioprocesses arises from the many potential applications of NSC technology, which include transplantation, gene therapy, drug delivery, drug development and testing, and biological research (Fig. 2). Many of these applications require large numbers of cells or processes that can generate cells, but under strictly controlled conditions.

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Treatment of Neurodegenerative Diseases. Neurodegenerative disorders, such as PD and Huntington's disease (HD), are conditions in which specific cell populations within the CNS degenerate and die. PD is characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) region of the brain resulting in severe motor and mental dysfunctions. Although drug therapy with L-3,4-Dihydroxyphenylalanine (L-DOPA) relieves some of the symptoms of PD, its benefits are usually limited to 3–5 years due to continued loss of DA neurons and development of disabling dyskinesias (involuntary, uncontrollable, and often excessive movement). HD is an autosomal dominant genetic disorder caused by repeats of the trinucleotide sequence CAG in the IT15 gene (codes for the protein huntingtin) located near the telomere of the short arm of chromosome 4 (21). The physical manifestation of this mutation is the eventual degeneration of striatal gamma amino butyric acid (GABA) producing cells, leading to disinhibition of the thalamus and brainstem, involuntary choreiform (dancelike) spasmatic movements, personality changes, progressive dementia, and eventually death (22). The exact mechanism by which cell death occurs remains unknown (23). The administration of pharmacological treatment (including neurotrophic factors) has had little or no effect on the symptoms of this disease, and has not prevented the progression of HD (24,25).

One approach to treating PD is to compensate for the loss of essential neurons by replacing them with functional cells. In the late 1970s and throughout the 1980s, many fetal tissue grafting experiments were conducted in animal models (reviewed in Ref. 26). On the basis of the encouraging results in the laboratory (significant graft survival, synapse formation, graft induced-dopamine release, and behavioral recovery), the procedures were clinically adapted for humans. Originally, adrenal medullary grafts from the afflicted individual were transplanted into the nigral region. The rationale for this was that by using autologous tissue, ethical and immunological concerns surrounding the use of fetal tissue could be avoided. However, follow-up studies have revealed only a modest improvement in 30% of treated individuals. Thus, the clinical use of primary fetal ventral mesencephalic (VM) tissue for transplantation was initiated in the late 1980s. Since that time, at least 350 patients have undergone neural tissue transplantation worldwide (27). Although the results have generally been favorable from a clinical standpoint (most individuals have shown marked improvement in function), relief of symptoms was sometimes incomplete, and occasionally motor complications were observed (reviewed in Refs (26,28–31)). The variable outcomes observed in clinical trials of fetal tissue transplantation may be related to differences in cell survival associated with different methods of tissue preparation and surgical transplantation procedures (32). Recent advances in these areas, which resulted in the development of a novel transplantation methodology, have demonstrated that patients who received fetal midbrain-derived cell transplants (as a cell suspension) had favorable clinical evolution and did not develop motor complications (33,34). Postmortem analysis of these patients revealed that the cell grafts integrated seamlessly into the Parkinsonian brain were not rejected by the host, and restored the cell type that provides specific DA innervation to the most affected striatal regions.
Those studies indicate that optimizing transplant methodology may enhance clinical outcomes, and in conjunction with almost three decades of accumulated experimental and clinical neural transplantation, it is clear that cell restoration is feasible and perhaps the most promising therapeutic strategy for PD.

On the basis of the success with fetal cell transplantation, alternative to primary fetal tissue. Many studies have initiated limited clinical trials to assess the efficacy of treating HD patients with transplanted fetal striatal tissue. Stereotaxic injection was used to implant fetal tissue into the striatum of patients (35–37). It was shown that implanted cells can efficiently integrate into host tissue (38), and can result in motor and cognitive improvements in recipients (39,40). Although these are positive and significant findings, the clinical improvements may not be stable over the long term (41). This may be linked to the tissue procurement procedures and surgical techniques used, which as shown for PD, had a significant effect on patient outcome (33). As such, it is clear that cell transplantation strategies are effective in treating neurodegenerative conditions, but much work remains to be done before it is approved for widespread use in a clinical setting.

Although the clinical use of fetal tissue for transplantation shows promising results for the treatment of neurodegenerative disorders, there are several concerns that must be addressed. First, there are moral and ethical concerns associated with using fetal tissue, which could present a serious obstacle to this type of treatment. Secondly, although 70–80% of the transplanted cells are viable, only 5–10% of the tissue survives at the implant site (42). Thus, to ensure that sufficient numbers of cells survive and become functional in vivo following transplantation, it is necessary to collect and implant cells from several fetuses for each treatment procedure. With more than a million Parkinson’s patients in North America, many millions of aborted fetuses would be necessary to provide treatment for all of the patients. Obviously, this is not feasible. Thirdly, the cost of isolating fetal tissue and subsequent surgical transplantation is enormous, and could prove to be inhibitory to this form of treatment. Finally, since the fetal tissue is not derived from a single source, it is not well-characterized. Typically, the cell populations used come from elective abortions and not spontaneous abortions, since those from the latter have a higher incidence of chromosomal abnormalities. However, they could still contain microbial contaminants or cancerous cells (42).

NSCs expanded in culture represent an attractive alternative to primary fetal tissue. Many studies have demonstrated that NSCs transplanted into animal models of neurodegenerative disease can migrate, proliferate, and integrate into host tissue (discussed in Refs 43 and 44). These cells have been shown to alleviate many of the functional deficits associated with neural disease by repopulating regions of absent or dead cells. Hammang and colleagues (45) transplanted fetal epidermal growth factor (EGF) responsive stem cells into a myelin deficient rat. The stem cell progeny were found to differentiate preferentially into oligodendrocytes in response to the nonmyelinated CNS, and subsequently produce myelin. Svendsen and colleagues (46) transplanted progenitor cells isolated from the developing human CNS into rats with unilateral DA lesions. After 20 weeks, differentiated graft-derived neurons were found close to the transplantation site, and human astrocytes were found to have migrated throughout the striatum. In a few of the animals, a significant number of new neurons were dopaminergic, and it was found that they were able to reverse rotational deficits associated with the lesion. Freshly isolated human NSCs have been successfully transplanted into the brains of immune deficient neonatal mice (47), and human NSCs grown in vitro for 1 year have been shown to migrate and integrate into the developing rat brain (48). Bromodeoxyuridine-labeled human NSCs have also been transplanted into the developing nonhuman primate brain where they were found to integrate into both the mature cerebral cortex and the subventricular zone (49). In addition to the reports discussed here, transplantation studies are routinely used to determine if cells cultured in vitro can survive, integrate, and function in vivo. The cells are typically labeled with a marker such as Green Fluorescent Protein (GFP), and functionality is assessed by anatomical, electrophysiological, and/or behavioral studies. Human NSCs transplanted into a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse brain were shown to be able to migrate away from the transplantation site and survive for at least 90 days (50). Moreover, McBride and colleagues (51) have shown that human NSC transplants directly into the CNS improved motor function in a rat model of HD, and Lee et al. (52) demonstrated that it was possible to induce functional recovery in HD through intravenous administration of NSCs.

Most recently, we have shown in a collaboration with colleagues at the Cell Restoration Laboratory in Halifax (Canada) that human NSCs expanded in suspension culture can survive transplantation into animal models of neurodegenerative disease, differentiate to a specialized neural cell type, and incorporate into the architecture of the brain without causing tumor formation (53). Moreover, bioreactor-expanded human NSCs, differentiated to a GABAergic phenotype, were shown to have efficacy in reversing symptoms associated with PD (54,55), HD (56), and spinal cord pain (allodynia) (57) in animal models.

Other Neural Stem Cell Applications. NSCs have a multitude of other applications in addition to direct cell replacement. The first of these is further research. Although many studies have already been published in this area, much research remains to be conducted in order to study and generate effective methods to tailor the proliferation, migration, and differentiation of NSCs. One difficulty in this area is comparing studies from different research laboratories since cells from different sources vary in their behavior. NSCs generated in a single large-scale facility could be distributed to laboratories throughout the world so that all of these research facilities could have uniform cells with which to conduct research. In order to address the heterogeneity between cell types, this facility...
could use standard protocols to expand cell populations procured from several different sources.

NSCs could also be used to test novel therapeutic drugs. Currently, animal models are extensively used to test the efficacy and potential hazards of new medicines. Although animal models have been proven to be acceptable in this arena, the effect of these drugs on animal cells is not identical to the effects on human cells. Indeed, it is often the case that the production and disbursement of particular drugs have been halted due to unforeseen side effects that did not occur in animals. Cultured NSCs and their progeny would allow researchers to directly observe the impact of new drugs on human neural cells, thereby enabling the production of safer and more effective medicines. In addition, it may be possible to isolate bioactive products of therapeutic interest from NSCs.

A problem with indirectly delivering therapeutics to the CNS is the blood–brain barrier. This barrier tightly regulates the passage of compounds into the CNS. Thus, in order to ensure that adequate quantities of a drug are being supplied to the CNS, it is often necessary to produce drugs that have a long half-life and to prescribe high doses of those drugs. To solve this problem, NSCs could be used as drug delivery vehicles to supply specific doses of certain therapeutics directly into the CNS. Cells could be genetically manipulated to produce neurotrophic factors, neurotransmitters, and other active compounds, and then transplanted into desired regions within the CNS. In this way, it would be possible to supplement chemicals that are produced at subeffective levels or replace chemicals that are not present due to cell death or genetic mutation. For example, Kordower and colleagues (58) isolated EGF-responsive stem cells from transgenic mice. These cells were genetically modified to express human nerve growth factor (hNGF) under the direction of the Glial Fibrillary Acidic Protein (GFAP) promoter. These GFAP-hNGF cells were then transplanted by intraocular injection into a rat model of HD. The study found that the cellular delivery of hNGF by genetically modified stem cells prevented the degeneration of striatal tissue otherwise destined to die. The ability of transgene-expressing NSCs to act as therapeutic vehicles in the treatment of brain disorders is further enhanced by their extraordinary ability to migrate large distances (59). For example, a study by Aboody and colleagues (60) showed that when genetically modified NSCs were implanted into intracranial gliomas in adult rodents, the NSCs distributed themselves extensively throughout the tumor bed, and migrated uniquely in juxtaposition to expanding tumor cells while continuing to express a foreign gene. When the cells were implanted intracranially at some distance from the tumor, the cells migrated through normal tissue and again targeted the tumor cells. In addition to drug delivery, NSCs could also be genetically manipulated to direct their own differentiation, or the differentiation of surrounding precursor cells toward certain desired phenotypes (e.g., DA neurons).

NSCs grown in vitro could be used to further understand aspects of neural development. Specifically, studies could be conducted to understand the underlying mechanisms that control the lineage and ultimate fate of different groups of neural precursor cells, including the identification of mediators and inductive factors in neurogenesis. These cells could also be used to create models of neurodegeneration to gain further knowledge about the causes and progression of neural disorders. Finally, the cells could also be used in the rapidly emerging fields of genomics and proteomics to understand how genetic programs within cells change due to differentiation, and how they are affected by autocrine and paracrine factors.

**Advantages of Neural Stem Cells over Primary Fetal Tissue.** NSCs cultured in vitro in controlled bioreactors may be a viable alternative to primary fetal NSCs, without the same issues. First, the cells could be grown efficiently in large quantities in bioreactors. This would alleviate current and future supply problems for large number of potential applications, and would reduce overall costs. Secondly, the moral and ethical concerns associated with harvesting large quantities of fetal tissue would be significantly reduced. Thirdly, since large quantities of cells could be produced from a single source, the cells would be well characterized. By setting up appropriate screening protocols, it could be ensured that the cells are nontransformed, nontumorigenic, and free of adventitious agents.

**SCALE-UP OF STEM CELL CULTURE**

SSCs (i.e., adult stem cells) are found at a frequency of 0.01% or lower in hematopoietic tissue (hematopoietic stem cells) (61) and 0.2–0.4% in the adult forebrain subependyma (NSCs) (62). In order to meet the needs of the many patients affected by neurodegenerative diseases, and to overcome the small cell numbers associated with stem cell availability in fetal or adult tissues, the generation of many cells in a controlled process is required.

Cell culture can be defined as adherent, where cells grow attached to a culture substrate (i.e., dish, T-flask, and microcarrier) or nonadherent, where cells grow as suspended single cells or aggregates. In addition, nonadherent culture can be further divided into stationary, where the culture medium is static, or suspension, where the culture medium is agitated or perfused. NSCs typically grow as suspended spherical aggregates (i.e., nonadherent stationary culture), and can be induced to grow as spherical aggregates in a stirred-tank bioreactor (i.e., nonadherent suspension culture) (63,64). There have been cases where researchers have cultured NSCs as adherent monolayers, but that type of growth is difficult to scale up as the cell growth surface needs to be scaled up as well (65). This is difficult and labor intensive if one uses tissue culture flasks or cell factories. In addition, one might consider using microcarrier cultures, but this is technically very challenging. In addition, any adherent culture process has a major disadvantage in that the cells must be removed from the surface for further culturing, a process that undoubtedly damages them.

**Stationary Culture**

NSCs in stationary culture are inoculated as a dispersed single-cell suspension, typically between $10^5$–$10^6$
cells/mL. Depending on the cell culture medium used, doubling times range from 12–20 h (murine) to 4–6 days (human). As the stem cells proliferate, they remain attached to one another, forming spherical aggregates. The exact mechanism of aggregate formation is not entirely characterized, but we have recently determined that human NSCs attach to the culture dish soon after inoculation, and then migrate to form small colonies after a few days, which then lift off from the culture surface to give suspended aggregates of cells. NSCs are typically passaged every 4 (murine) or 14 days (human) by breaking up the spherical aggregates and reinoculating a new flask with a single-cell suspension.

In order to generate large numbers of cells, large volumes of medium are required, as the maximum cell density achievable is limited by factors such as oxygen delivery (66). For example, the oxygen consumption rate of murine NSCs is $3 \times 10^{-17} \text{mol/cell} \cdot \text{s}$, and the volumetric mass transfer coefficient ($k_{La}$) through the culture medium surface - flask - is $1.53 \text{ h}^{-1}$ for a T-25 with 6 mL of medium. Given that the maximum solubility of oxygen in cell culture media is 0.22 μmol/mL, a simple calculation will show that the maximum sustainable cell density is $3 \times 10^6$ cells/mL based on oxygen supply. To reach higher total numbers of cells in stationary culture T-flasks, large culture volumes are achieved by increasing the number of T-flasks—there are commercially available models with a surface area of up to 300 cm², which would be able to hold a volume of 60 mL of medium and cells. To hold 5 L, this would require 84 T-flasks that would have to be individually inoculated, counted, and maintained (Fig. 3).

This is labor intensive and would also result in 84 different batch vessels. Alternatively, a single 5-L bioreactor could generate at least $5 \times 10^9$ cells in a single run.

**Suspension Culture**

NSCs in suspension culture are also inoculated as single cells, which form small aggregates during the initial stages of the culture. These aggregates then grow by cell division throughout the remaining culture period to form larger aggregates. Mass transfer of nutrients and oxygen to cells within these aggregates is through the process of diffusion via the void spaces between cells. This mass transfer process, coupled with the consumption of the nutrient/oxygen, results in an exponentially decreasing concentration profile through the aggregate from the outer edge to the center. As oxygen has a low solubility in media, it is important to maintain small aggregate sizes, so that nutrient/oxygen limitations do not occur at the centers of large aggregates. In extreme cases (very large aggregates), cell death occurs due to necrosis. As is outlined below, we have developed methods to control the diameter of our NSC aggregates below levels where necrosis could occur (67), and have developed correlations to show that the medium viscosity and agitation rate can directly impact aggregate size (68).

**Scale-Up Issues**

In order to use expanded cell populations in clinical applications, it is necessary to ensure that the expansion protocols and procedures being employed are standardized, and result in reproducible outcomes. However, the development of such bioprocessing methods requires many issues...
to be addressed (Fig. 4). Those issues can be categorized as pertaining to (i) cell culture medium, (ii) cell handling procedures, and (iii) bioreactor protocols, all of which will be individually addressed in this article. It should be noted that all suspension bioreactor experiments reported in this article were conducted in 125-mL spinner flasks (diameter = 0.065 m) with a working volume of 100 mL. Following inoculation, the flasks were incubated at 37°C in a humidified atmosphere containing 5% CO2. The agitation rate in the spinner flasks was controlled at 100 rpm by a calibrated magnetic stir plate and a 0.052-m diameter impeller suspended above the bottom of the flask (69).

**STEM CELL CULTURE MEDIA**

The purpose of a cell culture medium is to provide an optimal liquid environment in which isolated cells can survive, grow, function, and divide. Developing a medium that will eventually be used for the large-scale expansion of NSCs is challenging for two reasons. First, the medium has to be able to sustain the growth of stem cells for a long period of time in culture while allowing them to maintain their defining characteristics. Secondly, the cost of the medium must be minimized so that large quantities of cells can be generated using an economically viable process. Indeed, one of the disadvantages of performing fetal tissue transplantation to treat neurodegenerative disorders is that it is cost prohibitive. Since cell culture medium is a significant expense in mammalian cell culture processes, optimization studies have to be conducted to generate a cost-effective medium.

A number of cell culture media have been reported in the literature for the expansion of NSCs. However, the most commonly used medium reported in the literature was Normal Murine (NM) medium (17), which was developed for the expansion of murine NSCs. It is composed of equal amounts of Dulbecco’s modified Eagle’s medium (DMEM) and Hams F12 supplemented with various components, including insulin, selenium, progesterone, transferrin, putrescine, and EGF. Although this medium was found to be adequate at expanding murine NSCs, both the expansion rate and efficiency of sphere production were low, and the medium was not capable of supporting the long-term expansion of human NSCs. Sen and Behie (70) reported a new medium capable of supporting higher expansion rates for murine NSCs. Subsequently, metabolic studies were carried out to optimize this new medium, including evaluating the impact of nutrient levels, growth factors, lipids, hormones, trace elements, and vitamins as well as evaluating impact of physicochemical properties such as pH and osmolarity. The resulting medium, named PPRF-m4, was found to be capable of supporting long-term expansion of murine NSCs in both stationary and suspension culture (Figs 5 and 6). The cells retained their ability to proliferate, self-renew, and differentiate into the three primary cell types found in the CNS (67). Moreover, this medium was capable of supporting the expansion of human NSCs in tissue culture flasks (Fig. 5d). Through a number of optimization studies, PPRF-m4 has evolved to PPRF-h2, a new medium that can support the rapid expansion of human NSCs in suspension culture without compromising their stem cell properties. Cells expanded in this medium have shown efficacy in reversing symptoms associated with PD, HD, and alldynia in animal models (55,56,57).

**STEM CELL HANDLING PROTOCOLS**

**Cell Passaging**

NSCs grow as aggregates, which reach large diameters near the end of batch culture runs. There are many methods available to break apart the aggregates to obtain a single-cell suspension, including mechanical, enzymatic, and chemical means. Traditionally, NSCs have been mechanically broken up (i.e. tituration)—this process has several steps: (i) collecting the cell suspension
Figure 5. A new medium developed in our laboratory has the capacity to expand both murine and human neural stem cells. (a) Viable cell density and viability for cell lines W1, W2, and W3 expanded in NM medium. The cells were inoculated at 75,000 cells/mL into T-25 flasks containing 5 mL of NM medium. The flasks were then incubated at 37°C in a humidified chamber containing 5% CO₂. (b) The cell density and viability of murine neural stem cell line W3 inoculated into NM medium and PPRF-m4 medium. (c) The cell density and viability of cell lines W1, W2, and W3 serially passaged in PPRF-m4. The cells were inoculated at 75,000 cells/mL into T-25 flasks containing 5 mL of medium. The flasks were then incubated at 37°C in a humidified chamber containing 5% CO₂, and passaged every 4 days. (d) The viable cell density, total cell density, and viability of human neural stem cells (cell line M006) being serially passaged in PPRF-m4. The cells were inoculated at 200,000 cells/mL into T-25 flasks containing 5 mL of PPRF-m4 medium. The flasks were then placed in a 37°C incubator with a humidified atmosphere containing 5% CO₂. The cultures were fed with 2 mL of fresh medium on days 4, 8, and 12, and an equal volume of spent medium was removed. After 14 days, the human cells were passaged into fresh PPRF-m4 medium. The experiments were conducted in duplicate, and the error bars represent the range of values obtained. The lines on each graph go through the mean of the obtained data at each time point (83).
in centrifuge tubes, (ii) centrifuging to obtain a cell pellet, (iii) removing the supernatant, and (iv) repeatedly drawing up and then expelling the cell pellet through the opening of a micropipet, while pressing against the bottom of the centrifuge tube. This creates a single-cell suspension as a result of the high shear forces experienced by the cells during the repeated passes through the narrow opening of the pipet tip. However, this harsh method can result in the death of up to 50% of the cells. Many different enzymes, including trypsin, collagenase, dispase, and others, are available for cell and tissue dissociation. They work by cleaving membrane-bound and extracellular proteins. It has been shown that these are quite harmful to NSCs, when used in high enough concentrations to cause cell dissociation. To overcome this problem, we have developed a novel chemical dissociation procedure to create a single-cell suspension in a matter of minutes, while minimizing cell death and maintaining high cell viabilities (Fig. 7). In addition, batch-to-batch variation in mechanical dissociation procedures caused by different users, pipet aspirating procedures, and pellet sizes have been removed, resulting in a reproducible, standard protocol that can easily be scaled up.

Cryopreservation
Following cell expansion, it may be necessary to cryopreserve cells for a period of time before they are used in a particular application. Thus, determining the effect of cryopreservation on the cells and developing effective cryopreservation methods is necessary. We have compared different methods of freezing on the cell density and viability of murine NSCs (Table 1), and based on post-thaw cell survival, freezing NSC aggregates in PPRF-m4 +10% dimethyl sulfoxide was the preferable cryopreservation approach amongst the methods evaluated (Fig 8a). In addition, repeated freeze–thaw cycles (Fig 8b), and the length of time NSCs were frozen (Fig. 8c) did not have a significant impact on the cell density and viability.

BIOREACTOR PROTOCOLS
Inoculation
The process of bioreactor inoculation requires careful optimization of conditions to ensure maximum cell survival (75). An important parameter is inoculation cell density. In general, lower inoculation cell densities result in larger population amplifications, but require longer culture periods to reach maximum cell densities. This is evident in Fig. 9, where we show that murine NSC cultures inoculated between $1 \times 10^3$ and $1 \times 10^5$ cells/mL have vastly different cell densities after 4 days (increasing with increasing inoculation density) (Fig. 9a), but the amplification of cell population follows the reverse trend (decreasing with increasing inoculation density) (Fig. 9b). Analysis of the growth curves shows that the exponential phase growth rate also depends on inoculation cell density with extremely low and high inoculation densities causing a reduction in the growth rate (Fig. 9c). Large cell numbers use up nutrients quickly, and low cell numbers do not rapidly generate adequate concentrations of necessary paracrine factors following inoculation, thereby negatively impacting cell proliferation rates. The maximum amplification of cells, when left to reach their peak cell density, is higher at low inoculation densities. NSCs, as they grow in the form of aggregates, have an added level of complexity, as it is necessary to break up the aggregates prior to inoculation to ensure a
homogeneous culture, as discussed above. We have found that our chemical dissociation protocol works very well at generating inoculum for batch cultures and for serially passaging cells for an extended period of time (Fig. 7).

Agitation

The agitation rate influences two major stem cell culture variables: the shear experienced by the cells and the mass transfer coefficient of oxygen delivery from the headspace gas to the bulk liquid medium. As in many cell culture processes, these variables are in conflict. A high level of mass transfer (high agitation rate) and a low level of shear forces on the cells (low agitation rate) are desired. NSC cultures require agitation rates that maintain the aggregates in suspension, and create shear levels that control aggregate diameter without causing damage to the cells. We have previously examined this in great detail for the murine NSC system (66–68,72). Importantly, we have found that the diameter of NSC aggregates (both the mean and maximum of the population) can be controlled below necrotic levels using medium design and shear control (Fig. 10).

Oxygen

Many bioreactor processes for mammalian cells, when scaled up to larger volumes, require higher levels of oxygen delivery that can be provided through surface aeration alone. This is owing to the decrease in the surface area to volume ratio that usually occurs upon scale-up. Solutions to this problem include enriching the headspace gas for oxygen (>21% O2), increasing the agitation rate, sparging with air, or switching to a bubble column or air-lift bioreactor (outside the scope of this article). As is the case with many stem cell systems, using knowledge from developmental biology, and in this case, neurobiology, can provide clues for optimizing bioprocesses. The in vivo environment in a developing embryo has very low levels of oxygen, and in the case of NSCs, the oxygen level also has a signaling role in vivo (16,17). Lower levels of oxygen have also been linked with increased proliferation of NSCs in static culture (73,74). In suspension culture bioreactors, we examined the effect of hyperoxic (35% O2) (Fig. 11a) and hypoxic (5% O2) (Fig. 11b) levels of oxygen in the headspace gas versus the standard 21% O2. Hyperoxic conditions resulted in slower growth rates, and lower total and viable cell densities. The higher cell viabilities observed were most likely due to further penetration of oxygen into the NSC aggregates. Conversely, hypoxic conditions resulted in lower viabilities, most likely due to oxygen limitations at the centers of NSC aggregates. However, it also resulted in higher growth rates, and higher total and viable cell densities. This highlights the need for careful study of parameters, such as oxygen level, in stem cell cultures as they may need to be maintained at levels other than those routinely used for general mammalian cell cultures.

Production Modes

Bioreactor processes can run in batch, fed-batch, and perfusion modes. Although batch cultures are simpler to operate, they have several disadvantages from a stem cell bioprocessing point of view. First, cells in batch cultures can be exposed to changing pH, nutrient levels, and signaling concentrations, which may cause unwanted differentiation or loss in viability. Secondly, the cell composition changes significantly during a batch culture, and can impact the final cell population. For example, a buildup of differentiated cells may have a negative effect on the proliferation of the stem cell subpopulation. Fed-batch cultures are better than batch cultures at maintaining desirable growth conditions since nutrients can be replaced. However, perfusion cultures, in which fresh medium is added and spent medium is removed, can provide a homeostatic growth environment most similar to that found in vivo.

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Table 1. The Six Methods Evaluated for Neural Precursor Cell Cryopreservation

<table>
<thead>
<tr>
<th>Method</th>
<th>Cryopreservation Medium</th>
<th>Cryopreservation Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PPRF-m4</td>
<td>Aggregates</td>
</tr>
<tr>
<td>2</td>
<td>PPRF-m4 + 10% DMSO</td>
<td>Aggregates</td>
</tr>
<tr>
<td>3</td>
<td>PPRF-m4 + 10% DMSO</td>
<td>Single cells</td>
</tr>
<tr>
<td>4</td>
<td>PPRF-m4 + 10% DMSO</td>
<td>Single cells</td>
</tr>
<tr>
<td>5</td>
<td>PPRF-m4 + 10% glycerol</td>
<td>Aggregates</td>
</tr>
<tr>
<td>6</td>
<td>PPRF-m4 + 10% glycerol</td>
<td>Single cells</td>
</tr>
</tbody>
</table>

*DMSO, dimethyl sulfoxide.
Extended Culture

Newly developed media and bioreactor protocols are usually tested in short batch cultures for mammalian cells. However, stem cell populations take time to adapt to new conditions, and the only true test of a stem cell is the retention of self-renewal and multipotency over an extended period of time in vitro. For this reason, extended cultures (greater than 10 passages) must be performed using the
Figure 9. Inoculation density is known to have a significant effect in certain mammalian cell cultures. (a) Effect of inoculation density on measured cell density and viability in stationary cultures of murine neural stem cells. Single cells were inoculated at various densities into T-25 flasks containing 5 mL of PPRF-m4 medium. The flasks were then placed in a 37°C incubator with a humidified atmosphere containing 5% CO₂ for 4 days. (b) Effect of inoculation density on the multiplication ratio and cumulative number of doublings for the results shown in (a). (c) The effect of inoculation density on the subsequent measured cell density in suspension culture. Single cells were inoculated at various densities into 125-mL spinner flasks containing 100 mL of PPRF-m4 medium. The flasks were then placed in a 37°C incubator with a humidified atmosphere containing 5% CO₂. The agitation rate was controlled at 100 rpm using a magnetic stir plate. The spinner flasks were sampled daily. (d) The effect of inoculation density on the maximum measured multiplication ratio and number of doublings in suspension culture for the results shown in (c). The experiments were conducted in duplicate, and the error bars represent the range of values obtained (63).
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Figure 10. Effect of shear (agitation rate) on maximum aggregate diameter of murine NSCs in batch suspension bioreactors. All data points represent the average of duplicate spinner flasks each counted twice. Error bars represent the standard error for each measurement (66).

Figure 11. Oxygen levels in culture were found to have an impact on murine neural stem cell expansion. (a) The effect of 35% oxygen (hyperoxic conditions) on cell proliferation and culture viability in spinner flasks as compared to cells grown in a control culture with 21% oxygen (from air) (normoxic) and (b) the effect of 5% oxygen (hypoxic conditions) on cell proliferation and culture viability in spinner flasks as compared to cells grown in a control culture with 21% oxygen (from air). The peak average viable cell density (day 5) in (b) was statistically higher than that in the control culture. In both (a) and (b), single cells were inoculated into 125-mL spinner flasks containing 100 mL of PPRF-m4 medium. The flasks were incubated at 37°C in a humidified atmosphere containing 5% CO2. The agitation rate was controlled at 100 rpm using a magnetic stir plate. The spinner flasks were sampled daily. The experiments were conducted in duplicate, and the error bars represent the range of values obtained (63).
growth factors should result in a cessation of cell prolifera-
tion. The ultimate test of NSC performance is transplantation in an animal model, where migration, integration, differ-
entiation, and a lack of tumor formation are the desired outcomes.

CONCLUSIONS AND FUTURE DEVELOPMENTS

The development of stem cell expansion media, cell han-
dling procedures, and bioreactor protocols has enabled the successful generation of large quantities of NSC popula-
tions in suspension bioreactors, first with mouse cells and more recently with human cells. As the field of regenerative medicine moves toward the clinic, the requirements for cells and reagents used in clinical settings will become more rigorous. This means that defined media and process validation will become important. Ultimately, the need for controlled, robust systems for the production of stem cells and their progeny will require bioprocesses designed with traditional biochemi-
cal engineering expertise and a thorough understanding of the complexities of stem cell biology.

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