

# Bringing Neural Cell Therapies to the Clinic: Past and Future Strategies

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**Cell replacement therapy in the nervous system has a rich history, with ~40 years of research and ~30 years of clinical experience. There is compelling evidence that appropriate cells can integrate and function in the dysfunctioning human nervous system, but the clinical results are mixed in practice. A number of factors conspire to vary patient outcome: the indication, cell source, patient selection, and team performing transplantation are all variables that can affect efficacy. Most early clinical trials have used fetal cells, a limited cell source that resists scale and standardization. Direct fetal cell transplantation creates significant challenges to commercialization that is the ultimate goal of an effective cell therapy. One approach to help scale and standardize fetal cell preparations is the expansion of neural cells in vitro. Expansion is achieved by transformation or through the application of mitogens before cryopreservation. Recently, neural cells derived from pluripotent stem cells have provided a scalable alternative. Pluripotent stem cells are desirable for manufacturing but present alternative concerns and manufacturing obstacles. All cell sources require robust and reproducible manufacturing to make nervous system cell replacement therapy an option for patients. Here, we discuss the challenges and opportunities for cell replacement in the nervous system. In this review, we give an overview of completed and ongoing neural cell transplantation clinical trials, and we discuss the challenges and opportunities for future cell replacement trials with a particular focus on pluripotent stem cell-derived therapies.**

## Introduction

The nervous system is a large and complex tissue that controls many bodily functions such as sight, smell, sound, feel, taste, voluntary and involuntary muscle movements, autonomic functions, and our ability to reason. It has been estimated that hundreds of different types of neurons are precisely wired together and supported by glial and other support cells types to achieve tissue function.<sup>1</sup> During development, the nervous system originates from a simple sheet of neural stem cells that assembles into a complicated, three-dimensional structure over time. By birth, the neural stem cells have largely differentiated into neurons and glia and cannot be replaced if lost due to injury or disease. This leaves only a few small pools of neural stem cells (NSCs) that provide limited renewable neuron types in specialized brain regions. A focal loss of neurons usually compromises the function of the injured brain region, leading to a distinct clinical presentation. And while glial cells have some capacity to aid regeneration, many

diseases occur due to glial degeneration itself or the inability of glia to repair widespread neuronal damage. The goal of cell therapies in the nervous system is to manufacture neural cells that can be surgically transplanted and will integrate and function to mitigate the patient's symptoms. Conceptually, there have been many different approaches to restore brain function through cell replacement therapies. They fall largely into three categories: (1) proliferation or transdifferentiation of resident cells, (2) fetal or adult stem and progenitor cells, and (3) human pluripotent stem cell-derived cell types. The recent work in transdifferentiation of non-neuronal cells into neuronal tissues is fascinating but beyond the scope of this review. Here, we will focus our discussion on using fetal or pluripotent-derived neural cells to manufacture cell therapies.

## Pioneering Fetal Cell Studies

The original clinical transplantations using fetal neural tissues occurred in the late 1980s for Parkinson's disease (PD) and late 1990s for Huntington's disease (for review, see Lindvall and Björklund,<sup>2</sup> Brundin et al.,<sup>3</sup> and Barker et al.<sup>4</sup>). In each disease, the degeneration of a small, discrete population of neurons underlies a majority of their distinct clinical symptoms, and animal models had provided proof of concept for cell replacement. While the efficacy of the therapies is controversial, there is little doubt that the lack of standardization in cell source and process made the outcomes of this therapy less robust. Between the different studies, investigators varied the number of grafted cells, used different methods to preserve tissue derived from the multiple aborted fetuses required, and varied the immunosuppression regimen, with some groups electing to forgo immunosuppression entirely. These differences notwithstanding, there have been case reports showing improved dopaminergic function after transplantation and survival of grafted cells for up to 24 years,<sup>5</sup> and a meta-analysis of the collective data has shown that fetal cell transplantation can be effective for certain patient populations.<sup>6</sup> The variability of transplantation included side effects called graft-induced dyskinesias, involuntary movements that were not predicted by animal models of disease.<sup>7,8</sup> The increased regulatory burden on such direct fetal transplantations has made them more difficult to perform,

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although a European research consortium called TRANSEURO (led by Roger Barker) has recently performed fetal cell transplantations in PD patients to test the efficacy of the method with the benefit of hindsight.<sup>9,10</sup> These important studies should help guide future transplantation paradigms for PD.

#### **Working with Limited Source Material: Neural Stem Cell Expansion**

A limitation of fetal cell transplantation is the small amount of tissue available, even from large numbers of fetuses: some transplantation paradigms require up to ten aborted fetuses per patient treated (for example, see Kefalopoulou et al.<sup>11</sup>). This presents logistical complications in terms of coordination of tissue donation and also raises ethical concerns. One way to overcome this limitation is to expand neural stem or progenitor cells, thus reducing the amount of starting material required. Like all stem cells, neural stem cells have the capacity to both self-renew and differentiate into specific progeny; they are the building block of the nervous system.<sup>12,13</sup> Resident stem or progenitors can be directly isolated from a developing fetus before expansion in the mitogen fibroblast growth factor 2 (FGF2) and, for later cells, epidermal growth factor (EGF).<sup>14,15</sup> Stem and progenitors can expand either as “neurospheres” (floating aggregates) or as adherent cultures. Removal of mitogens then allows stem cell differentiation into neurons or glia. Expansion increases cell number but complicates the biology while increasing regulatory scrutiny. Most regulatory agencies change the status of the transplant from a simple tissue to a more manipulated product once expansion has occurred.

Numerous studies over the years have examined neural stem and progenitor expansion, but two problems are sometimes encountered: (1) loss of “patterning” and (2) reduced production of neurons and increased glial cell production. Neural stem and progenitor cells retain a cellular memory of the regional identity from which they were derived, but this can be lost over passage (e.g., Studer et al.<sup>16</sup>). There is evidence that the stage of embryonic development used to initiate cultures can influence whether NSCs retain their regional identity over passage.<sup>17</sup> In our opinion, deriving specific regional neuronal tissues from NSCs remains a challenge, and no universal neuron will be able to fulfill the exquisitely specialized function of a neuronal subclass. Expanded cells can also lose neurogenic capacity as the stem and progenitor cell divides, mirroring development: neural stem cells first primarily produce neurons but switch to glia cells as development proceeds. This “developmental clock” appears to remain functional *in vitro*, potentially confounding manufacturing NSCs. One notable exception are the so-called long-term neuroepithelial stem cells (Lt-NES).<sup>18</sup> Lt-NES cells appear to lack the developmental clock (making them “long-term”) and as such are ideal candidates for manufacturing. Lt-NES cells adopt a hindbrain regional identity and have some flexibility in patterning, but the extent of this plasticity remains to be determined. Taken together, the evidence suggests that NSC expansion can be helpful for manufacturing and cryopreservation of a cell product, but there are several key aspects that need further inves-

tigation and careful scrutiny to verify that the product does not “drift” during expansion.

#### **Review of Completed and Ongoing Clinical Trials with Neural Stem Cells**

Over the last several decades, many biotechnology companies have formed to develop and manufacture neural cell therapies. Largely, they were based on academic findings and provided valuable insights into the use of neural cell types for replacement therapies, such as route of administration, animal models, and regulatory pathways. Table 1 summarizes selected clinical trials as listed on <https://www.clinicaltrials.gov> that are relevant to this review. We limited this review to therapies using human neural cells that have the potential to be manufactured for extended clinical use.

##### **Layton BioScience: LBS Neurons**

Early attempts at manufacturing neural cells occurred before the discovery of human embryonic stem cells and at the dawn of NSC biology. A cell line called Tera-2 was derived from a human teratocarcinoma, a cancer of the reproductive organs containing embryonic-like cells. Derivative subclones were demonstrated to have pluripotent differentiation capacity after transplantation.<sup>19</sup> These cells could be expanded indefinitely due to their cancerous nature, making them ideal for manufacturing. Expansion can be stopped and differentiation initiated through exposure to retinoic acid.<sup>20</sup> Later work showed that a specific subclone (called NT2/D1) behaved like a committed neuronal precursor, narrowing the normal pluripotent cell fates to the desired dividing “progenitor” and “neuron” depending on growth conditions.<sup>21</sup> A company called Layton BioScience manufactured these cells as a product (LBS Neurons) and led them through phase 1 and 2 clinical trials.<sup>22,23</sup> These trials used immunosuppression, and some evidence was presented that cells survived in patients for at least a few years, although it is difficult to judge the extent of survival.<sup>24</sup> The number of patients treated was small and the results too variable to draw any positive efficacy conclusions, although no adverse reactions were thought to be caused by the cells. While there were many positive reports of function in animal models, some found that the cells incompletely differentiated and did not function in animal models of disease (for example, see Fricker-Gates et al.<sup>25</sup>). It appears that Layton BioScience is no longer advancing NT2-based cells for clinical utility. While it is difficult to imagine gaining regulatory approval to transplant a cancer-derived cell line with major karyotypic abnormalities today, LBS Neurons are relatively homogeneous and the differentiated state could be cryopreserved, allowing for ease of manufacturing, standardization, and quality control that are not available from primary fetal tissue.

##### **ReNeuron: CTX-DP (CTX0E03)**

Another manufactured neural cell product is a genetically engineered, conditionally immortalized neural stem cell line called CTX0E03 that was derived from 12-week human fetal cortical neuroepithelial tissue. Human cortical tissue was dissected and cultured before transformation with inducible c-mycER<sup>TAM</sup> delivered via a Moloney murine leukemia virus (MMLV) viral vector. Expansion and differentiation of

**Table 1. Clinical Trials Using Manufactured Cell Therapies for Neural Diseases**

NCT #	Title	Recruitment	Phases	Last Updated
<b>ReNeuron<sup>a</sup></b>				
NCT01151124	pilot investigation of stem cells in stroke	active, not recruiting	1	May 31, 2016
NCT02117635	pilot investigation of stem cells in stroke phase II efficacy	recruiting	2	June 21, 2016
NCT01916369	safety trial of CTX cells in patients with lower limb ischemia	recruiting	1	May 31, 2016
<b>Neuralstem<sup>b</sup></b>				
NCT01730716	dose escalation and safety study of human spinal cord derived neural stem cell transplantation for the treatment of amyotrophic lateral sclerosis	active, not recruiting	2	April 16, 2015
NCT01348451	human spinal cord derived neural stem cell transplantation for the treatment of amyotrophic lateral sclerosis	active, not recruiting	1	March 9, 2016
NCT01772810	safety study of human spinal cord-derived neural stem cell transplantation for the treatment of chronic SCI	active, not recruiting	1	August 24, 2015
<b>StemCells<sup>c</sup></b>				
NCT01321333	study of human central nervous system stem cells (HuCNS-SC) in patients with thoracic spinal cord injury	completed	1/2	June 16, 2015
NCT01632527	study of human central nervous system stem cells (HuCNS-SC) in age-related macular degeneration (AMD)	completed	1/2	September 10, 2015
NCT01725880	long-term follow-up of transplanted human central nervous system stem cells (HuCNS-SC) in spinal cord trauma subjects	terminated		June 1, 2016
NCT01238315	safety and efficacy study of HuCNS-SC in subjects with neuronal ceroid lipofuscinosis	withdrawn	1	January 13, 2015
<b>Geron/Asterias<sup>d</sup></b>				
NCT02302157	dose escalation study of AST-OPC-1 in spinal cord injury	recruiting	1/2	August 11, 2016
<b>International Stem Cell Corporation<sup>e</sup></b>				
NCT02452723	a study to evaluate the safety of neural stem cells in patients with Parkinson's disease (Cyto Therapeutics Pty Limited)	recruiting	1	March 10, 2016

<sup>a</sup>Conditionally immortalized human neural stem cell line (c-mycER<sup>TAM</sup>).  
<sup>b</sup>Expanded neural stem cell products derived from human fetus.  
<sup>c</sup>Expanded neural stem cell product derived from FACS-purified fetal neural stem cells.  
<sup>d</sup>First human embryonic stem cell-derived product in patients; oligodendrocyte precursor cell product.  
<sup>e</sup>Parthenogenetic embryonic stem cell-derived neural stem cell.

this product can be controlled by the application of 4-hydroxy-tamoxifen that activates the mycER(TAM) transgene.<sup>26</sup> This product was adapted to an automated, serum-free production system capable of producing 20 × T175 flasks.<sup>27</sup> A company called ReNeuron has led multiple clinical trials in different indications with CTX-DP (CTX0E03 prepared for transplantation; Table 1). They recently published positive safety data for their Pilot Investigation of Stem Cells in Stroke (PISCES) phase 1 study for treatment of stroke,<sup>28</sup> a dose-escalation design with single doses of 2, 5, 10, and 20 million cells injected into the putamen without immunosuppression.<sup>28</sup> To the best of our knowledge, no post-mortem studies have been published to provide evidence of cell survival after transplantation.

#### **Neuralstem: NSI-566**

Neuralstem manufactured a neural stem cell product called NSI-566. This product was initially isolated from the spinal cord of an 8-week-old human fetus. A single-cell suspension was created and expanded as an adherent culture in typical neural stem cell expansion conditions with FGF2.<sup>29</sup> The neurons created from expanded cells did not have the characteristics of motor neurons unless they were “primed” by exposure to factors thought to encourage motor neuron cell fate.<sup>30</sup> After priming, the differentiated cells created ~50% neurons and 50% glia, and the number of neurons with a motor neuron phenotype was ~15% (or 7.5% of total cells).<sup>31</sup> To date, NSI-566RSC has been transplanted in 30 patients with amyotrophic lateral



sclerosis (ALS) through two clinical trials and has met primary safety endpoints. Immunosuppression was used, but to the best of our knowledge, no post-mortem studies have been published to provide evidence of cell survival after transplantation.

Neuralstem's manufacturing scheme has been published. The line was initially derived in a research lab, but passage 4 cells were transferred into a good manufacturing practice (GMP) facility and used to construct a master cell bank (MCB) at passage 6, a working cell bank (WCB) at passage 9, and a clinical cell bank (CCB) at passage 12. From a total of 10 million cells at passage 4, Neuralstem was able to generate nearly 400 vials of cells at 16 million cells per vial at passage 12 using their GMP process. The company reports no change of the neuron-to-glia ratio over this passage range.<sup>32</sup> The reported expansion under GMP conditions is remarkable and allows one to theorize that this approach could be applied to a larger number of patients, although source material may at some point become limiting.

#### **StemCells: HuCNS-SC**

The HuCNS-SC product is an expanded human fetal forebrain NSC initially isolated by enriching for stem cells through flow cytometry. Irv Weissman, Fred Gage, and their colleagues had shown that human CNS stem cells could be prospectively enriched by sorting the CD133<sup>+</sup>/CD24<sup>-/LO</sup> population from fetal brain before neurosphere expansion.<sup>33</sup> StemCells used this paradigm to develop HuCNS-SC into a clinically compatible product intended to treat a wide variety of CNS disorders (for review, see Tsukamoto et al.<sup>34</sup>). Phase 1 and 1/2 trials were completed for Pelizaeus-Merzbacher disease, neuronal ceroid lipofuscinosis, spinal cord injury, and age-related macular degeneration. The product proved safe and integration was shown in some contexts,<sup>35</sup> but it failed to provide a "robust clinical result" according to chief executive officer (CEO) Ian Massey (<http://www.stemcellsinc.com>). The company began an orderly wind down and recently merged with an Israeli company called Microbot Medical that plans to develop robotics-based medical devices (<http://www.stemcellsinc.com>). As such, it seems unlikely that HuCNS-SC will play any clinical role going forward, although it is possible the product and intellectual property could be sold to another company to continue development.

#### **Geron/Asterias: OPC1**

Geron was the first company to advance an embryonic stem cell-derived product to the clinic. Their first cell product (GRNOPC1) is a characterized composition of cells with the active cell type being oligodendrocyte precursor cells for the treatment of spinal cord injury. Geron famously faced a great deal of regulatory scrutiny, with the CEO showing a ~28,000-page document required to move the cells to trial, one of the longest ever submitted to the US Food and Drug Administration (FDA).<sup>36</sup> The first cohort contained five patients that met the primary endpoint of safety in the phase I trial. Despite this, Geron decided to focus its resources on cancer and terminate the program.<sup>37</sup> In 2013, BioTime acquired the rights and product from Geron and re-initiated the project through a subsidiary

called Asterias Biotherapeutics. GRNOPC1 was rebranded as AST-OPC-1, and the product has now entered a phase 1/2a trial with help from the California Institute for Regenerative Medicine (CIRM). The initial Geron cohort treated five patients with a dose of two million cells, whereas the ongoing Asterias phase 1/2a is a dose-escalation study (10 million and 20 million cells). The second cohort is ongoing, with five patients already transplanted at the 10-million-cell dose at the time of writing.<sup>38</sup> The study initially planned to transplant 13 patients but is now seeking approval to transplant 40 (<http://asteriasbiotherapeutics.com>). "Low level" immunosuppression was used for 60 days to help cells resist rejection, although we are unaware of any post-mortem analysis showing survival and integration in patients to date. Taken together, these are encouraging results, as they demonstrate that a human embryonic stem cell (ESC) therapy can safely be brought into the clinic and that a composition of cells, rather than a single defined cell type, can be brought forward through the regulatory path.

#### **International Stem Cell Corporation: ISC-hpNSC**

The International Stem Cell Corporation (ISCO) has adopted an ESC-based platform, but with a twist: they use parthenogenetic ESCs. Human eggs are manipulated to create an identical copy of its haploid genome to make a dividing, homozygous diploid cell line. Homozygous cells have fewer human leukocyte antigen (HLA) alleles, reducing the immunogenicity of transplanted cells. The cell product called ISC-hpNSCs is directed to a "generic" (presumably forebrain fated) neural stem cell.<sup>39,40</sup> ISCO has entered a phase 1 trial for PD in Australia and recently reported transplanting their first patient.<sup>41</sup> As illustrated in this case, there are alternative pathways to seek regulatory approval for cell therapies, but a safe and efficacious therapy must be the primary goal of such studies.

It remains to be seen which of these therapies will become routine and compete against established therapies for the indication, a key aspect when one considers the commercial value of such products. A few of these product-indication combinations aim to replace the primary cell lost in disease, which will require specialized, subtype-specific neural cells to function. These therapies will require a deep understanding of the mechanism of action for the product to replace the very cell responsible for the pathophysiology. In contrast, most products described above hope to provide a supportive role to modulate the immune response and provide paracrine support to the damaged brain. These therapies have a more general mode of action and may be used in a spectrum of diseases where pro-survival factors are beneficial. The former are likely to show greater efficacy, but such products are generally more difficult to manufacture. It is important to remember that the number of cells that survive grafting can directly impact efficacy *and* safety. We believe the strength of immunosuppression can affect survival, even in the "immunoprivileged" nervous system. In the cases presented, only post-mortem analysis can provide definitive data for graft size and survival, so some of the presented safety and efficacy data must be interpreted with this caveat in mind. Moving forward, it will be important to define strategies to monitor graft size in vivo. In PD, for example, one can monitor



dopamine metabolism using positron emission tomography (PET) scans. These tests can then confirm the causality between the efficacious therapy and the assumed mechanism of action. Nevertheless, these studies showed that there is a path for clinical use of cell therapies to treat diseases of the nervous system.

### Starting Material

#### *Fetal and Adult Neural Stem and Progenitor Cells*

Neural stem and progenitor cells can be expanded either as adherent or suspension cells. Other strategies are a mixture of both, such as adhering to the surface of microbeads or trapped inside of a matrix. Adherent cultures are usually passaged enzymatically, but spheres are passaged either by enzymes or by physical cutting.<sup>42</sup> While chopping can be performed in a GMP facility,<sup>43</sup> it is generally a high-risk manipulation and difficult to perform at scale. A better approach could be to pass spheres through a “Biogrid,” a pressurized grid composed of micron-scale knife edges.<sup>44</sup> Scale up in a stirred bioreactor can provide large increases in cell number without using excessive amounts of surface area and media. Obstacles to obtaining healthy cultures in such systems include providing adequate oxygen exchange to the medium and the shear force imposed by such culture. High impeller speeds increase oxygen supply and create homogeneity of the culture environment but at the cost of raising shear force that can damage cells. Software control of the bioreactor can monitor and dynamically adjust important parameters of the culture, potentially allowing higher cell densities with better viability and quality while saving resources.<sup>45</sup> While most work for NSCs has been in suspension, alternative adherent strategies have also been pursued. One method is to grow cells in a hollow fiber bioreactor such as Quantum Cell Expansion System by Terumo and Xpansion Multiplate Bioreactor System or related technologies. A potential complication of such systems is that the cells are inaccessible morphologically and can be difficult to remove enzymatically. An alternative approach is to use an automated system that has been adapted to traditional flasks such as the Compact SC from TAP Biosystems. This system has been used to expand many stem cell types (in cell numbers up to  $10^{11}$ )<sup>27,46</sup> and was used by StemCells for at least some of their applications.<sup>47</sup>

#### *Pluripotent Stem Cells*

Because fetal tissue is limited and in vitro expansion can alter cell fate and potential, many groups (such as our own) have used human pluripotent stem cells as an initiating cell source for production. Pluripotent stem cells (PSCs) are made from two main sources: ESCs are derived from in vitro fertilized embryos, and induced pluripotent stem cells (iPSCs) are somatic cells that have been transcriptionally “rebooted” to a stem cell-like state through transient, ectopic expression of key pluripotent transcription factors (for review, see Takahashi et al.<sup>48</sup>). Under the right conditions, both types of PSCs are “naturally immortal” cells that divide rapidly without transformation and retain the ability to make all three germ layers of a developing embryo. Mouse PSCs can make every cell of the developing organism in tetraploid complementation studies.<sup>49,50</sup> Due to ethical concerns, one can only assume that human PSCs would also have the broad capacity to differentiate into any cell of the adult human being. Taken

together, these studies make PSCs an ideal candidate to be used as a starting material for cell therapies.

Generation of iPSCs is a laborious process, and if the starting material is an iPSC cell line, the process of its generation should be automated and standardized. This has recently been addressed using a custom-built automation platform. The New York Stem Cell Foundation developed liquid handling robots that enable the automation of deriving, characterizing, and differentiating iPSCs.<sup>51</sup> In contrast to manual derivation at the clonal level, the system prepares pools of iPSCs. The authors further show that the pools of iPSCs are stable over time, but it remains to be seen if this approach will be widely adopted.

Much effort has been invested into creating more defined, simple, and robust methods to expand PSCs. Automated culture and passaging of iPSCs using Compact Select, a tissue flask based automation platform, has been demonstrated by a few labs.<sup>46,52,53</sup> The authors identified several bottlenecks with the existing automation platform that require further refinements of the protocols and possibly the machinery (minimal size of pipettes and the time it needs for the cell clumps to settle). The limitation of adherent PSC culture can be overcome by the expansion of PSCs in stirred bioreactors,<sup>54,55</sup> although it is important to remember that this could affect their ability to differentiate. Some process development time might be required if the characteristics of the PSCs have changed because of the change in culture method.

#### **Generating Differentiated Progeny**

The challenge of using PSCs is to create robust protocols to direct them into the desired cell type, a process that is intrinsic to a developing embryo but difficult to recapitulate in vitro. The most successful protocols developed today thus mirror developmental processes and require the activation and/or blocking of different signaling pathways in a precise temporal sequence.<sup>56,57</sup> For neuronal cell therapies, an efficient conversion of PSCs into neuronal fates is essential. Directing PSCs to an early neuroectodermal fate has become highly efficient and synchronous since the development of the dual SMAD inhibition (DSi) protocol.<sup>58</sup> Altering additional signaling pathways during DSi can direct PSCs into different neuroectodermal-derived cell types such as neural crest<sup>59,60</sup> (which can further differentiate into melanocytes<sup>60</sup> and sensory neurons<sup>59</sup> among others), placodes (which can further differentiate into pituitary cells<sup>61,62</sup> among others), and many types of central nervous system (CNS) neurons and glial cells. Select examples of CNS neurons made using DSi include hypothalamic,<sup>63</sup> forebrain,<sup>64–66</sup> spinal motor,<sup>67,68</sup> and midbrain dopamine neurons.<sup>69</sup>

Despite this progress, it can be challenging to completely direct all PSCs to a given cell fate. On the other hand, fetal tissue is also mixed: dissecting the ventral mesencephalon typically gives 10%–15% of the desired midbrain dopamine neurons in the best case. Some groups report much worse recovery, often as low as 2%.<sup>70</sup> However for many applications, it is unclear if a single cell type is the best product.



We believe that the main constituent of the product should be the cell type that mediates the function. Supporting cell types, such as glia, may provide supportive benefits such as secretion of growth factors or removal of cellular waste.<sup>71</sup> To this end, PSC differentiation protocols can be superior to NSC expansion methods, with PSC motor-neurons generating a purity of up to ~60%–70% in the best cases<sup>72</sup> (Elizabeth Calder, personal communication) versus 7.5% for NSC expansion.<sup>31</sup> Asterias's OPC1 product is composed of not a single cell type but rather a defined mixture of cells that may act in concert to mediate the effect. It will be important to truly understand the mechanism of action of the cellular therapy to decide what level of purity will be desirable or required for the cells to exert their clinical effect. One further needs to have well-characterized and validated quality control assays to (1) confirm the identity of the product or product composition and (2) account for any unwanted cell types, such as remaining PSCs or cells contributing to side effects, such as serotonergic cells in fetal cell transplants for PD.<sup>73</sup>

### Risks of In Vitro Expanded Cell Types

#### Potential for Tumor Formation

The “naturally immortal” nature of PSCs makes them a concern for transplantation. In fact, the gold standard to prove their pluripotency is to transplant them into an animal and allow them to form a teratoma, a tumor made of derivatives from all three germ layers. Methods to direct PSCs into specific cell fates must be robust, uniform, and unidirectional, since residual PSCs result in the risk of tumor formation. Even derivative cell types must be carefully controlled; we and others have found, for example, that so-called rosette-derived neural cells can create overgrowths in vivo<sup>74</sup> (unpublished data). It seems likely that some robustly dividing progenitors are capable of paracrine signaling to each other if transplanted at high enough density. These dangers are not specific to PSC-derived cells, since one of the only reports of a transplant-derived tumor came from a transplantation that used fetal cells as the source material.<sup>75</sup>

#### Karyotypic Abnormalities

One additional concern for using PSCs or extensively passaged neuronal stem and progenitor cells is that they can lose a normal karyotype over passage in culture. This does not necessarily mean that such cells would result in uncontrolled growth after transplantation (see above section Layton BioScience: LBS Neurons). But such abnormalities could affect many aspects of the cell's biology. It is important to use well-characterized, early-passage PSCs that have been thoroughly vetted as seed stocks for building subsequent master and working cell banks. It should be noted, however, that karyotypic abnormalities have been found during NSC propagation,<sup>76,77</sup> despite a reputation for being more genetically stable. It seems likely that expansion of any cell type provides a selective pressure for genetic variants that divide faster in culture, so genetic integrity should be monitored for any cultured cell product. One current challenge is that smaller genetic changes are often present in different human genomes

and create uncertainty with respect to what is considered “normal.”<sup>78</sup>

### Considerations for Manufacturing

In order for neural cell products to become a successful therapy that can be applied to all patients in need, a defined product for each specific indication needs to be manufactured at scale. While small molecules, and to some extent other biologics, can be manufactured in large quantities and at high purities,<sup>79</sup> cell therapies from pluripotent or multipotent cell sources are inherently more difficult to produce. A single culture condition will usually not suffice, so the process of manufacturing is ever changing and requires the introduction of many raw ingredients and multiple manipulation steps. Today, scale is mainly achieved by manual expansion through operator manipulation of traditional culture vessels, by automated culture in traditional vessels, or by the use of small bioreactors. The choice is dependent on many variables, such as the preference of the cell to grow in suspension or adherent, and the desire to visualize the cells during the process. Bioreactors can support suspension cultures and cultures of small cell aggregates, although the use of carrier beads provides a hybrid solution and can allow for the culture of adherent cells (for review, see Merten<sup>80</sup>). While many efforts have been made to produce biologics at large scale, manufacturing can be further complicated when using autologous material. Production for many patients then becomes a challenge of scale out and not scale up. Now the facility must ensure that each patient's cells are unmistakably separated from the other patient material processed in the same laboratory or plant. Regulatory approval optimally requires approval for the process of production and not the end product. This route provides less quality assurance and more variability in manufacturing, since the cell source is constantly changing; both attributes increase regulatory scrutiny and concern. In addition, processes that are scaled out do not benefit from the same cost savings as one imagines from a scale-up procedure.

#### Open versus Closed Culture Systems

Traditionally, cell culture is performed in open systems, but conversion to a closed system is preferred for manufacturing a cell therapy. Closed systems are not required for early-phase clinical trials by regulatory standards in the United States, but they provide advantages not present in open systems. Closed systems are designed such that the product is not exposed to the room environment. Starting material and reagents are introduced via a sterile port and may be filtered prior to addition. Since open systems are exposed to the room environment, they require equipment such as biosafety cabinets and clean room technology to minimize the risk of product contamination. However, developing a closed system is often product specific, and the cost associated with such a development is usually not warranted for a phase 1 investigational new drug. Early versions of a development candidate are thus often manufactured in open systems. A variety of supplies are now available that reduce the number of manipulation steps, such as multi-layer tissue culture flasks<sup>81</sup> that reduce the number of openings and closings of the vessel during



media transfer processes, bridging the difference between those two approaches.

Closed systems also allow for parallel processing of multiple cell products, which is especially important when products need to be manufactured in a patient-specific manner and the scale out is more important than the scale up.<sup>82</sup> Equipment manufacturers recognize this void in technology and are developing automated systems to manufacture diverse cellular products in closed systems. One such instrument (CliniMACS Prodigy by Miltenyi Biotec) can culture and process cells via centrifugation and magnetic cell sorting.<sup>83</sup> Robust and standard operating procedure (SOP)-driven differentiation protocols will be essential for such fully automated processes to be successful.

### GMP Reagents

Another challenge is access to high-quality, controlled reagents. Both maintaining stem cells and coaxing them to become terminally differentiated cell types requires the use of specific growth media, growth factors, or small molecules that provide cell fate and differentiation cues to manufactured cells. Many media formulations and supplements were initially developed for the culture of rodent brain tissue and cells prior to the advent of human cell therapies. Formulations such as Neurobasal, B27, and N2 have now been widely adopted for human cell culture. While this is not a problem for academic research, it poses a challenge for GMP production. Suppliers are now developing xeno-free materials and fully qualified reagents (such as the Cell Therapy Systems line of products from Thermo Fisher Scientific and other reagents from Miltenyi Biotec and R&D Systems). However, many of the supplies are still difficult to source, the product is extremely costly, or the biological activity of the new product has been changed or compromised. Swapping reagents midway through the product development cycle may alter the quality or quantity of the final product.

The process of manufacturing reagents under cGMP is neither trivial nor cheap, both in terms of time and money. Certain molecules are used across disciplines and the combined demand allows manufacturers to profitably produce GMP product. Reagents with a more narrow use case might not be manufactured under GMP conditions if they are deemed unprofitable. While most manufacturers will provide custom manufacturing to improve quality assurance, the cost can be prohibitive. This may require investigators to “qualify” a reagent by examining the manufacturing practices of non-GMP sources commercially available. GMP is a suite of regulations that ensures the quality and control of these supplies, but one can use alternative tools and approaches to justify the use of a given reagent in early-stage clinical trials.<sup>84</sup> The investigator can thus use non-GMP reagents and qualify them for a phase 1 production. In most cases, however, sourcing GMP-ready material should be the preferred process. It should also be noted that the “GMP” designation is interpreted in different ways by different companies, so due diligence should be applied to all reagents regardless of claimed manufacturing status.

### Outlook: The Advantages and Disadvantages of iPSCs versus Human ESCs

Based on the shortcomings of primary sourced material and transformed cell lines we believe that human PSCs hold the greatest potential for future neural cell therapies. The first human ESC therapies have entered the clinic, and several new therapies are being prepared for testing in clinical trials.<sup>85</sup> The derivation of iPSCs in recent years has opened a novel therapeutic angle, that of matching the replacement tissue to the recipient. While the CNS is often thought to be an immunoprivileged site of the body, it is clear that allogeneic tissue grafts can be recognized by the immune system and sometimes rejected or chronically attacked at a lower level.<sup>86</sup> Most groups performing allotransplants use at least temporary immune suppression to avoid graft rejection, but there is significant evidence that grafts can persist for many years without immunosuppression (for review, see Freed et al.<sup>87</sup>). The available evidence could be interpreted to suggest that the CNS has a muted immune response rather than absolute privilege. Autologous tissues for replacement therapies would be advantageous, since grafted cells could better evade immune surveillance.

There was significant concern about the clinical use of iPSCs, since early methods used retroviral vectors to deliver the reprogramming factors. These concerns have been somewhat mitigated by elegant methods that use “footprint-free” technologies, such as episomes, microRNA, or protein. Nevertheless, there is ample evidence that the reprogramming process itself induces or selects for rare mutations present in the parental somatic cells.<sup>88–93</sup> Double-stranded breaks (marked by gamma H2AX) are observed after the ectopic expression of reprogramming factors, and an intact homologous recombination-DNA (HR-DNA) repair pathway is required for reprogramming.<sup>94</sup> This fits with the observation that p53 suppression enhances reprogramming (for review, see Ebrahimi<sup>95</sup>). Cells damaged during reprogramming are lost through apoptosis and are a major contributor to the low efficiency of reprogramming.<sup>96</sup> Taken together, the available evidence suggests that reprogramming causes DNA damage<sup>94</sup> that is repaired through HR-DNA repair.<sup>94</sup> Removal of p53 genome surveillance improves reprogramming efficiency by allowing DNA damaged cells to avoid apoptosis.<sup>96</sup> These theoretical concerns became tangible during a recent clinical trial initiated in Japan for the treatment of macular degeneration. The goal was to use autologous iPSCs, but this strategy was halted after a mutation was discovered by deep sequencing of the derived cells in preparation for the second patient.<sup>97</sup> It remains unclear if the mutation was present in the donor cells, but it raises concerns that such rare mutations could be selected for during reprogramming and might provide additional risk once placed in a different cellular context after transplantation. This study and others following have now adopted an allotransplantation strategy that will use well-characterized iPSC banks.<sup>98</sup>

For all studies using cultured cells, it will be important to understand if such mutations actually present a safety risk. It is important to remember that people are routinely transplanted with lenti-integrated cells, in some cases for many years, with no adverse events reported.



GlaxoSmithKline now markets Strimvelis in Europe, a lenti-based gene transfer system that provides immunity to children with inherited immune disease (severe combined immunodeficiency/adenosine deaminase deficiency-severe combined immunodeficiency [ADA-SCID]).<sup>99</sup> These systems cannot control viral integration site, so thoughtful leaders in the gene transfer field have been carefully mapping the safety of disrupting all regions of the genome, a concept known as safe harbor.<sup>100</sup> The gene transfer field should remind us that modification of the genome is not necessarily dangerous. Our own history should provide additional reassurance, since LBS Neurons are karyotypically abnormal cells that nevertheless met their primary safety endpoint. While caution is warranted before transplanting cells, we must provide rational controls on cell products that do not unnecessarily impede progress. “Safety switches” that can inducibly kill grafted cells are another tool designed and evolved by the gene transfer field that are beginning to be implemented into PSCs that can provide additional safety measures.<sup>101</sup>

Other aspects of iPSCs and individualized medicine are the cost and time required. For the therapy to be applied to individuals, individual iPSCs must first be generated, expanded, and characterized before they can be used as source material for differentiated cells. Each therapy thus becomes a unique individual cell therapy, and one must prove that this process and product is reproducible from patient to patient. Derivation cost aside, this characterization will be very expensive unless the process becomes much more robust, obviating current regulatory requirements. Time plays another critical role, as such an individual iPSC therapy will be need to be manufactured on demand and cannot be an off-the-shelf product. In diseases where rapid intervention is required, a long waiting period may not be possible or desired. It would easily be several months before an autologous treatment would become available, pending no failed production runs. There are also cases where a patient suffers a genetic disease that would make their own cells less efficacious or more susceptible to the disease being treated.

An alternative approach is the generation of banks of pluripotent stem cells derived from so-called universal donors who are monoallelic at their major histocompatibility (MHC) loci and are thus suitable donors for an extended pool of recipients. Depending on the ethnic region, it is estimated that between 50 and 150 donor cells could serve the vast majority of the population. For example, a collection of iPSCs representing the most frequent HLA in each population would cover 78% of European Americans but would leave out 37% of Asians, 48% of Hispanics, and 55% of African Americans.<sup>102</sup> While such banks could be made as ESC banks, it is far more likely that they will be made as iPSC banks, due to the extended time it would take to collect suitable donor oocytes. Efforts are underway in Japan to cover most of their population, but many more banks would be required to treat the diversity of a country such as the United States and at a tremendous economic cost. Nevertheless, these large banks are now being constructed by many organizations for future clinical use.<sup>98,103,104</sup>

## Conclusions

In summary, PSC technologies offer an exciting avenue for CNS and peripheral nervous system (PNS) cellular repair that in many aspects is superior to the collection of adult or fetal material as a starting cell source. Because of their broad therapeutic potential, technologies to automate and optimize generation and differentiation will undoubtedly be developed, and with scale and demand, the cost per application will become more affordable. The cost and time required to develop a cell therapy should become less of an issue in the future, analogous to the evolution of genome sequencing technologies over the last few decades.

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