Differentiation of neural lineage cells from human pluripotent stem cells

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Accepted 25 March 2008
Available online 29 May 2008

Abstract

Human pluripotent stem cells have the unique properties of being able to proliferate indefinitely in their undifferentiated state and to differentiate into any somatic cell type. These cells are thus posited to be extremely useful for furthering our understanding of both normal and abnormal human development, providing a human cell preparation that can be used to screen for new reagents or therapeutic agents, and generating large numbers of differentiated cells that can be used for transplantation purposes. Critical among the applications for the latter are diseases and injuries of the nervous system, medical approaches to which have been, to date, primarily palliative in nature. Differentiation of human pluripotent stem cells into cells of the neural lineage, therefore, has become a central focus of a number of laboratories. This has resulted in the description in the literature of several dozen methods for neural cell differentiation from human pluripotent stem cells. Among these are methods for the generation of such divergent neural cells as dopaminergic neurons, retinal neurons, ventral motoneurons, and oligodendroglial progenitors. In this review, we attempt to fully describe most of these methods, breaking them down into five basic subdivisions: (1) starting material, (2) induction of loss of pluripotency, (3) neural induction, (4) neural maintenance and expansion, and (5) neuronal/glial differentiation. We also show data supporting the concept that undifferentiated human pluripotent stem cells appear to have an innate neural differentiation potential. In addition, we evaluate data comparing and contrasting neural stem cells differentiated from human pluripotent stem cells with those derived directly from the human brain.

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Keywords: Human pluripotent stem cells; Human embryonic stem cells; Human neural stem cells; Differentiation

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1. Introduction

It is widely appreciated that the central nervous system (CNS)\(^1\) has little capacity for self-repair after loss of cellular elements due to disease or injury. Indeed, it has long been held that any restoration of lost neurological function is primarily due to the subjugation of that function by intact redundant, parallel, and/or contralateral nervous pathways \([10,19,22,35,52,73,91,97]\). CNS diseases and injuries, therefore, represent a huge class of human maladies that have largely not been fully treatable using conventional medical approaches. Although progress has been made in the pharmacological treatment of some CNS diseases, such as depression, Parkinson’s disease, and phenylketonuria, most of the approaches taken are less than robust in their overall outcome or do little to address the underlying cause of neurological dysfunction. Despite these advances, many CNS diseases and injuries have little to no therapy available. These include traumatic injury, neurodegenerative diseases, and stroke. Among the obstacles to devising effective therapies for CNS disease and injuries are: a lack of knowledge about the basic pathophysiological mechanisms involved; methods for the timely evaluation of new therapeutic drugs and devices; a suitable transplantable cell population; and the very simple fact that the CNS is orders of magnitude more complicated than any other organ system. Much of the transplantation research directed toward devising therapies for CNS diseases and injuries has been based on neural tissue derived from the fetal brain or neural cells derived from neuroectodermal tumors \([20,29,34,44,119]\). Neither of these sources is entirely satisfactory. Therefore, alternative sources of transplantable material are needed.

The dawn of an entirely new approach to CNS diseases and injuries was heralded by two seminal discoveries: (1) there exists within the human brain a population of harvestable undifferentiated neurogenic cells (neural stem cells, NSCs, or brain-derived NSCs, bNSCs \([36,96]\)) and (2) a population of pluripotent stem cells (PSCs) with virtually unlimited proliferation potential and proven neurogenic potential can be harvested from the very early human embryo (embryo-derived pluripotent \([16,94,114]\) stem cells, ePSCs). The former discovery strongly suggests that there actually exists within the brain an innate repair mechanism and that medical interventions based on such a mechanism might be possible. The latter suggests that there exist readily replenishable sources of cells for such medical interventions. Therefore, a new and much invigorated effort has ensued to devise therapies for CNS diseases and injuries. Differentiation of ePSCs down the neural lineage, in fact, was one of the earliest described potentials of the PSC. Given the two basic properties of the PSC, therefore, an entirely new line of research has emerged: the use of the PSC to generate a replenishable population of neural cells not only for transplantation but also as a tool for drug and new therapeutic drugs and devices; a suitable transplantable cell population; and the very simple fact that the CNS is orders of magnitude more complicated than any other organ system. Much of the transplantation research directed toward devising therapies for CNS diseases and injuries has been based on neural tissue derived from the fetal brain or neural cells derived from neuroectodermal tumors \([20,29,34,44,119]\). Neither of these sources is entirely satisfactory. Therefore, alternative sources of transplantable material are needed.

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Three preclinical examples (among many) serve to illustrate the breadth of the transplantation effort and the breadth of possibility of the clinical efficacy of transplanted stem cells or their derivatives:

1. Use of neural cells differentiated from human ePSCs to treat an animal model of Parkinson’s disease \([98]\).

In this study, dopamine-producing neural cells differentiated from ePSCs were injected into the lesioned parenchyma of rats whose striatal tissue had been lesioned with 6-hydroxydopamine to produce a rat model of Parkinson’s disease. The implants gave rise to a long-lasting restitution of motor function and transplanted cells engrafted as tyrosine hydroxylase-positive neurons. Thus, trans-
planted cells gave rise to bona fide, functional, albeit ectopic, neuroregeneration.

(2) Use of neural cells differentiated from ePSCs or harvested from human brain to treat an animal model of a lethal lysosomal storage disease [62].

In this study, human neural cells were injected into the lateral cerebral ventricles of newborn mice that carried a lysosomal enzyme mutation producing the mouse-equivalent of the lethal human lysosomal storage disorder, Sandhoff’s disease. The primary goal of the study was to use the cells not to regenerate tissue or replace dead cells in the brain, but to efficiently deliver the missing enzyme to as large an extent of the brain as possible [102]. The study showed that a single intracerebroventricular cell injection, on the day of birth, significantly prolonged the lives of these mice and, at the same time, delayed the onset of the loss of motor skills. The mechanisms by which this neuroprotection occurred appeared to be manifold and included decreasing neuroinflammation as well as increasing the local activities of the defective enzyme. That mouse NSCs, human bNSCs and human ePSC-derived neural stem cells (eNSCs) had virtually identical efficacies and that the observed numbers of donor-derived differentiated neurons and glia were quite low supports the premise that the primary mechanism may be neuroprotection rather than neuroregeneration [102].

(3) Use of neural cells differentiated from ePSCs to treat an animal model of spinal cord injury [78].

In this study, oligodendroglial progenitors, differentiated from ePSCs, were implanted into the area of injury of adult rats whose spinal cords had been contused—a rat model of human spinal cord injury. The implanted cells caused an improvement of motor function in these animals and enhanced remyelination in the general area of the injury/implant. The implanted progenitors differentiated in situ into oligodendrocytes and induced an increase of oligodendrocyte remyelination, much of which was directly due to the implanted cells themselves. Thus, this example could be seen as a combination of neuroprotection and neuroregeneration as the progenitors replaced those lost in the host during the injury process and both remyelinated host axons and induced remyelination by the host, protecting those axons from secondary degeneration, one of the known sequelae to the injury [51]. The human PSC, therefore, because of its capacity for neural differentiation and because of its potential wide application to CNS therapeutics and basic research has received considerable attention from neuroscientists. As a result, there are a number of reports of methodologies for neural differentiation of this unique cell. Because of the plethora of neural cell types, most particularly neuronal cell types, the methodologies reported vary widely in their composition [8,9,11,31, 37,43,45,49,59,61,63,67,68,76,81,84,93, 100,101,108,116,122]. The purpose of this review is to comprehensively present the extant methodologies in a neurodevelopmental, stepwise manner, starting with the PSC and ending with the differentiated neural cell. The methodological steps, five in all, will be presented one at a time, comparing and contrasting the various methodological approaches at each step. The ultimate goal, therefore, is to give the interested reader a comprehensive, but organized, overview of the extant neural differentiation methodologies (summarized in Fig. 1 and Table 1) so that appropriate methodologies pertinent to their particular interests can be more easily appreciated and applied. A detailed methodology can be found in our recently published stem cell manual [39,64,82,109].

2. Description of methods

2.1. Human pluripotent stem cells

Most current lines of human PSCs have been obtained from the inner cell mass (ICM) of supernumerary embryos produced by in vitro fertilization (IVF [113]). In this procedure, eggs are harvested from a
Table 1
Methods for neural differentiation of PSCs

<table>
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<tr>
<th>1) Starting Material</th>
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<td>Collagenase/Mechanical</td>
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<td>3 days</td>
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<td>TrypLE Express</td>
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<tr>
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Table 1 (continued)

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<td>Yellow spheres</td>
<td>rosettes</td>
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woman after she has been treated with follicular hor-
mones to stimulate the ovaries. The eggs are fertilized
either by combining them with sperm in a dish or by
mechanically injecting the sperm into the egg (intra-
cytoplasmic sperm injection or ICSI). The fertilized eggs
are incubated to allow them to progress to the blasto-
cyst stage of embryonic development. At the blastocyst
stage, the trophectoderm of the embryo is removed
and the ICM is plated on to a “feeder layer” of mouse or
human embryonic fibroblasts [114], which are essen-
tial for the survival of the ICM [28]. The ICM then
flattens into a compact colony which is mechanically
dissociated and replated several times to eventually give
rise to a stable cell line of ePSCs.
ePSCs in culture have a specific morphology, and they express characteristic surface antigens and nuclear transcription factors. The colonies are very compact with many tightly spaced cells of large nucleus-to-cytoplasm ratios. The surface antigens include the stage-specific embryonic antigens SSEA-4 and SSEA-3 and the teratocarcinoma recognition antigens TRA-1-60 and TRA-1-81. Specific transcription factors that are associated with the expression of particular elements of the pluripotent genome include the POU (pit-oct-unc)-domain transcription factor Octamer-4 (Oct-4), Nanog, and Sox2.

When undifferentiated ePSC colonies are detached from the feeder layer and transferred into serum-containing medium, they form multicellular aggregates called embryoid bodies (EBs) which can contain cell types representing all three germ layers of the body—endoderm, mesoderm, and ectoderm. Many EBs tend to show cell types of only one or two germ layers, but in an unpredictable manner. Thus, with appropriate subculture conditions and physical removal of colonies showing specific morphologies, behaviors, or proteins, it is possible to establish cultures that are enriched for particular cell types or mixtures of cell types. However, this cell behavior is unpredictable and the sorting is not completely effective. Many labs have therefore attempted to develop protocols for directly controlling the differentiation of ePSCs.

Exogenous differentiating factors have been useful in favoring differentiation into specific derivatives: retinoic acid and nerve growth factor for neuronal differentiation; basic fibroblast growth factor and platelet-derived growth factor for glial precursors; 5-aza-2'-deoxycytidine for cardiomyocytes; bone morphogenetic protein-4 and transforming growth factor-β for trophoblast cells; sodium butyrate for hepatocytes; and various cytokines for hematopoietic cells. Differentiation into particular tissue types can also be elicited by over-expressing genes encoding transcription factors that function in cell commitment during normal development: MyoD1 for skeletal muscle; and Nurr1 for dopamine-producing neurons. However, these methods still usually only give enrichment rather than total induction, so additional sorting is often necessary. This has been performed based on lineage-specific gene expression: PS-NCAM and A2B5 as cell-surface markers for neural precursors; or hygromycin resistance driven by a myosin heavy chain promoter for cardiomyocytes.

Several groups have produced neuronal precursors from either mouse or human ePSCs and tested them following injection into the developing brain of newborn mice or rats. The transplanted cells were incorporated into the host brain, migrated along appropriate tracks, differentiated into neurons in a region-specific manner and made synaptic contacts with host neurons. In some cases the transplanted cells also gave rise to glia and astrocytes. This procedure has been shown to promote recovery in animal models of Parkinson’s disease and spinal cord injury.

Four other sources of PSCs that meet all of the above criteria have recently become available. The first is the blastomere. At the cleavage stage (8–16 cells) of embryonic development, a single cell (blastomere) is removed and placed in co-culture with ePSCs. The blastomere then divides and develops into a blastomere-derived PSC colony (bPSCs). In addition, parthenogenically-derived PSCs (pPSCs) have been described. In this case, single human eggs are stimulated, by incubation in low oxygen and application of calcium ionophores, to replicate their DNA and then divide to form a PSC colony. Next, there are PSCs derived from spermatogonial tissue (sPSCs). While these have been described in the literature for mice, the methods for the derivation of human cells have not yet been published. Finally, there are the induced PSCs (iPSCs). In this case, somatic cells are genetically transduced to express once-silent genes that reprogram these cells into PSCs directly, without going through a blastocyst stage. Two recent publications have demonstrated this with human cells. In one case, the cells were transduced to express Nanog, Oct4, Sox2, and Lin28. In the other, c-Myc, Oct4, Sox2, and Klf4 were required for the reprogramming. The iPSCs are of particular importance as they provide a potential source of patient-specific cells for transplantation as well as the opportunity to create PSCs for study from cells of patients with specific diseases. In addition, these cells do not have ethical concerns associated with the production of PSCs from human embryos.

2.2. Inherent neural potential of human ePSCs

PSCs appear to be an artifact of tissue culture, albeit a useful one. That is, no bona fide in vivo representation of the PSC has yet been convincingly described. Among their many unique properties, PSCs appear to be cells on the very verge of differentiating. Indeed, reliably maintaining PSCs in their undifferentiated state has been one of the central challenges of PSC culture. Even in their “undifferentiated” state, however, PSCs express a number of mRNAs that are specifically found in particular, more differentiated cells. Thus, PSCs, as a population, express a number of different multi-potent stem cell markers: nucleostemin and nestin, found in neural stem cells, for example. It is not yet clear, however, whether particular subpopulations of undifferentiated PSCs singly express a lineage specific set of stem cell markers or whether all PSCs express all stem cell markers (or some iteration between these two extremes). Moreover, undifferentiated PSCs also express mRNAs specific to more fully differentiated cell types. Thus, PSCs, as a population, express the mRNAs for neurofilament H, dopamine receptor 4, and doublecortin, which are neuronal markers; and for peripheral myelin protein and myelin-associated glycoprotein, which are glial markers. Again, it is not clear if subpopulations or the population as a whole are responsible. What is clearly suggested by these data, however, is that there is a relatively short distance between the gene expression state of
PSCs and the gene expression state of their more differentiated progeny—PSCs appear to be on the verge of differentiation and only the operation of a particular pluripotent expression program keeps them from differentiating.

Whether or not there is protein expression that corresponds to the mRNA expression described above has not been explored to any great extent. A simple experiment done in our laboratory, however, seems to show that, at least for some neuronal markers, not only is the protein expressed, but some cells show morphological and functional evidence of neuronal maturation in conditions that favor the undifferentiated state of PSCs. Thus, small, round, doublecortin-positive cells were found at the juncture of two colliding PSCs colonies (Fig. 3) and doublecortin-positive cells with morphologies exactly the same as those described for cells cultured from the human brain [103] were found outside the PSC colony in the surrounding MEFs (Fig. 3). This strongly suggests that, even under conditions that favor the pluripotent state, functional, maturing neural cells can develop, as doublecortin is known to be associated with migratory neuroblasts [38]. Although other cell types have also been shown to spontaneously differentiate from PSC colonies, none have been associated with a convincingly functional state. Thus, neural differentiation appears to be a lineage pathway easily accessible to PSCs under conditions commonly used to maintain these cells. This is certainly not surprising, given that the anlage of the nervous system, the neural tube, is one of the first tissues to form in the developing embryo.

ePSCs, therefore, can be considered the most immature human cell type that can be cultured for an extended period of time. The gene expression patterns in ePSCs are unique when compared to any other cell type that has been isolated and cultured so far. The last 2 years have seen a surge of landmark papers which have advanced our phenomenological knowledge of ePSCs by taking advantage of the latest developments in microarray and sequencing.
technologies. At least one common theme appears to emerge from these studies: the ePSC’s singular position within the “cell kingdom” depends upon seemingly opposite transcriptional characteristics: expression of most lineage fate genes that are known from differentiating cells is strongly repressed \([6,17,18,71]\) while, at the same time, most other genes from differentiated and, surprisingly, non-coding elements and regions in the eukaryotic genome are actively transcribed \([4,41,70]\). At the moment, this paradox can be only explained with teleological, high level concepts, since our ability to mechanistically dissect global system functions has yet to catch up with the pace at which we can phenotype cell types.

The current models propose that ePSCs are defined by a “bivalent” state on many levels of transcriptional control. These cells are “about to” readily differentiate, so every differentiation fate (which means the transcription of differentiation-inducing or -defining transcripts) is readily available, but also under tight, repressive control, since when “unleashed”, these genes and transcriptional elements will invariably tip the equilibrium towards differentiation and thus terminate the pluripotent state. When differentiation occurs, expression levels of specific, developmental fate-related genes can be rapidly up-regulated. The ability to access and transcribe other regions necessary for other developmental paths is, at the very same time, severely restricted. Stochastic resonance theory provides another interesting view of this low level transcription of most genetic elements in ePSCs: adding background noise to signals in unstable systems can better define their context and lead to more robust fate decisions \([14,15]\). It is important, therefore, not to rely on the expression of single, or even a few, genes or proteins as differentiation markers, but to attempt to measure the readout quantitatively and include proper controls for comparison. Most importantly, any system theory analysis of this process must follow function, not just markers.

As mentioned above, there appears to be a “default” mechanism for neuronal differentiation. We believe this observation needs further scrutiny, however; “defaultness” may be also a function of current PSC culture technologies, the small “developmental distance” between PSC and neuroepithelial cells, and a higher level of in vitro robustness of neural progenitors. The continuity of in vitro phenotypes when ePSC differentiate into neural stem cells appears more relevant to us. The recent work on inducing pluripotency in somatic cells suggests that a, yet undefined, “pluripotency program” is booted up into the “read-and-write memory” of the cells. This picture can help understand that, assuming the whole hypothetical regulatory network is not shut down all at once, there may be intermediate states, which could be restored to the starting point if the right signals are present in the culture medium. To what extent fates are reversible or cells can shift phenotypes is yet unknown. Recent work on rosette cells (see below) points towards much more plasticity of differentiated cells than previously assumed \([33]\), at least in vitro.

2.3. Overview of techniques (Fig. 1 and Table 1)

Fig. 1 schematically illustrates the overall organization of the methodologies used to differentiate PSCs down the neural lineage. In the first stage is the undifferentiated PSC. Since not all PSCs are cultured under similar conditions, the nature of this starting material will be described in some detail (Tables 1–3). In addition, newer culture media, with growth factors and co-factors already included, are radically changing the standard culture conditions for PSCs but there are as yet few manuscripts describing them so they will not be included here. During the second stage, the PSCs are induced to lose their pluripotentiality (We use this descriptor, rather than “induced to differentiate” as the method used is rather passive in nature and the latter term suggests a more intentional design.). Until recently, this step has consisted simply of depriving the PSCs of the feeder layer, culture substrate, and mitogens, thereby transforming them into the so-called embryoid bodies described above. While this method has its merits, some newer methodologies more fully direct the cells down the neural lineage rather than haphazardly down all three lineages simultaneously (see Table 1), although most of these methods result in initial lineages of more restricted potential. During stage 3, neural cells themselves are isolated and their fate becomes sealed by a specific change in culture conditions, similar to those used for brain-derived neural stem cells. During this stage, generally speaking, the cells still have the potential to generate any of the three basic lineages of neural cells—neurons, astrocytes, or oligodendrocytes. By stage 4, the cells’ fates have become more restricted: they are now fated to become either neurons or glia and by stage 5, a cell of restricted lineage is produced. In this stage, however, the cell may still be at the progenitor stage. If so, it is a progenitor for a single cell type.

2.3.1. Starting material (Table 1)

As stated above, all PSCs, to date, for which neural differentiation methodologies have been published, are ePSCs. The neural differentiation of at least 16 different ePSC lines has so far been described (Table 2). These lines include many on the NIH Stem Cell Registry as well as other more recently created lines. All described lines were either grown on irradiation- or mitomycin C-inactivated feeder cells or on Matrigel with medium conditioned by these feeder cells. Most ePSCs are mechanically passaged but some are passaged (as clumps of cells) after partial enzymatic digestion with trypsin, dispase, or collagenase. The culture media used for the various lines vary somewhat and the ingredients for those media are shown in Table 3. It should be noted that a recently published laboratory manual details the most common methods for ePSC culture and passaging \([39,109,110,118]\).

Despite the variation in culture conditions for the starting material, all the PSCs described show the same basic set of criteria: (1) morphology: compact colonies of small
phase-bright cells with high nucleus-to-cytoplasm ratios (Fig. 4); (2) gene expression: cells uniformly expressing Oct4, Nanog, Sox2, SSEA4, Tra-1-81; and (3) differentiation potential: cells generate tumors containing cells of endodermal, ectodermal, and mesodermal lineages. In addition, PSCs are routinely karyotyped to assure chromosomal stability as these cells can easily progress to aneuploidy. Finally, it should be noted that most cell lines, but not all, can be conditioned to thrive in any of the culture media described.

### 2.3.2. Induced loss of pluripotency (Table 1)

The most widely used method for inducing loss of pluripotency/inducing differentiation in PSCs is to enzymatically or mechanically lift the PSC colonies and place them, as clumps of cells, into low-adherence culture dishes or flasks without substrate, feeder cells, or mitogens. The exact media formulations vary (Table 1) but the formation of floating, spherical clusters or balls of cells of varying degrees and lineages of differentiation and known as embryoid bodies (EBs) is the

<table>
<thead>
<tr>
<th>Line ID</th>
<th>NIH registry cell line?</th>
<th>Culture medium (Table 3)</th>
<th>Substrate (Table 1)</th>
<th>Passage method</th>
<th>Derivation reference</th>
</tr>
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<tr>
<td>BG01 Y</td>
<td>STD</td>
<td>iMEF</td>
<td>Mechanical</td>
<td>[72]</td>
<td></td>
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<tr>
<td>BG01 Y</td>
<td>STD</td>
<td>mmcMEF</td>
<td>Mechanical</td>
<td>[72]</td>
<td></td>
</tr>
<tr>
<td>BG01 Y</td>
<td>STD</td>
<td>mmcMEF</td>
<td>Coll/Tryp</td>
<td>[72]</td>
<td></td>
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<td>BG01 Y</td>
<td>STD</td>
<td>mmcMEF</td>
<td>Collagenase</td>
<td>[72]</td>
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<tr>
<td>H1 Y</td>
<td>STD</td>
<td>iMEF</td>
<td>Mechanical</td>
<td>[114]</td>
<td></td>
</tr>
<tr>
<td>H1 Y</td>
<td>STD/CM</td>
<td>Matrigel</td>
<td>Collagenase</td>
<td>[114]</td>
<td></td>
</tr>
<tr>
<td>H7 Y</td>
<td>STD/Mod</td>
<td>mmcHFF</td>
<td>Collagenase</td>
<td>[114]</td>
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<td>H7 Y</td>
<td>STD/CM</td>
<td>Matrigel</td>
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<td>H9 Y</td>
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<td>Collagenase</td>
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<td>H9 Y</td>
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<td>H9 Y</td>
<td>Std</td>
<td>MMC</td>
<td>Collagenase</td>
<td>[114]</td>
<td></td>
</tr>
</tbody>
</table>
| Table 3 Various media formulations used in the culture of undifferentiated ePSCs (from Table 1)

<table>
<thead>
<tr>
<th>Medium Component</th>
<th>STD</th>
<th>STD–</th>
<th>STD/Mod</th>
<th>HES</th>
<th>HES/Mod</th>
<th>HUES</th>
<th>MB03</th>
<th>MB03/Mod</th>
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<tbody>
<tr>
<td>DMEM/F12</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>KO-DMEM</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>DMEM-HG</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>KO5R (%)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>–</td>
<td>20</td>
<td>10</td>
<td>–</td>
<td>20</td>
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<tr>
<td>FBS (%)</td>
<td>–</td>
<td>–</td>
<td>20</td>
<td>–</td>
<td>–</td>
<td>10</td>
<td>–</td>
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<tr>
<td>Plasmanate (%)</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>10</td>
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<td>IT5</td>
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<td>2×</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>2-ME (μM)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>55</td>
<td>55</td>
<td>100</td>
<td>100</td>
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<tr>
<td>NEAA (%)</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>1</td>
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<tr>
<td>GLN (mM)</td>
<td>1–2</td>
<td>–</td>
<td>1–2</td>
<td>–</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Ribonucleosides (%)</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Heparin (μg/mL)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>LIF (ng/mL)</td>
<td>–</td>
<td>–</td>
<td>2000 U/mL</td>
<td>–</td>
<td>12</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>bFGF (ng/mL)</td>
<td>4–20</td>
<td>4</td>
<td>40</td>
<td>–</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<tr>
<td>Noggin (ng/mL)</td>
<td>–</td>
<td>–</td>
<td>100</td>
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</table>
result. EBs, as a general rule, have been used as the source of partially differentiated cells for the production of a variety of tissue/cell types including neural cell types. EBs, even though the earliest differentiated progeny of PSCs, show a great degree of gene expression divergence from that of PSCs (Fig. 5) and immunocytochemical staining of EBs shows cells of all three germ lineages [12,32].

Non-EB approaches have also been used. The most common of these is the use of specific growth factor and/or antagonists to accelerate differentiation towards one cell type or lineage of interest (see Table 1). Other avenues of directed differentiation that have been employed involve the coculture of PSCs with cells of a particular origin that have been found to produce factors that enhance a specific lineage type phenotype. Future options may lie in the direction of specific lineage differentiation through the selective expression of specific transcription factors and/or combinations of transcription factors or through the use of small molecules that activate growth factor and or developmental pathways independent of the growth factors’ presence.

2.3.3. Neural induction (Table 1)

The EBs are differentiated to neuroepithelial cells in simple, serum-free culture media. EBs first are plated onto laminin-coated dishes to generate an adherent culture. The earliest reports of neural induction of EBs showed the formation of unique clusters of small, elongated (or columnar) cells surrounding a central, small, but cell-free zone. These neural rosettes [81,82], which resemble the morphology of the very early neural tube in cross-section, express early neural marker antigens such as nestin and Musashi-1, but not markers of more mature neural cells, which are found adjacent to the rosettes. Rosettes form the basis of most published methods of neural induction of PSCs. This method allows control of developmental stages and generation of primitive neuroepithelial cells which can be further induced to neuronal and glial progenitors with forebrain, mid/hindbrain, and spinal cord identities. Thus, this neuroepithelial differentiation method can be used to broadly generate neural progenitors and mature neural subtypes, and is well-adapted to the needs of individual investigators who intend to differentiate ePSC to specific types of neurons and glial cells. The protocol comprises three major steps: EB formation, differentiation of primitive neuroepithelial cells (i.e. neural rosette cells [33]), and generation of more definitive neuroepithelial cells. Each step is morphologically distinct and is readily identifiable under a regular phase contrast microscope. The typical yield of neuroepithelial cells, defined by immunostaining for the neuroepithelial transcription factors PAX6, SOX1, and SOX2, is about 90% of the total differentiated progenies [64,82].
2.3.4. Neural maintenance and expansion (Table 1)

The primitive neuroepithelial cells, or NSCs, are largely maintained and expanded in the presence of standard media containing growth factors. Basic fibroblast growth factor (bFGF) has been the predominant mitogen of choice [8,13,43,61,100]. Epidermal growth factor (EGF) [9,93], laminin [54] and ascorbic acid (AA) [61] have also been used in combination with FGF and other proprietary supplements. NSCs are commonly expanded as either rosettes or neural tubes on adherent substrates such as laminin, poly-ornithine (PLO), or FN, or as neurospheres (clonal clusters of cells) in suspension on low attachment polystyrene. Rosettes are typically expanded over a 7- to 10-day period prior to their isolation and plating for induction of terminal differentiation (Table 1). Neurospheres typically expand over the same time period; however, they are more amenable to passaging over several weeks. The neurospheres are dissociated into a cell suspension and replated under identical conditions. Here they may undergo another round of expansion prior to the induction of terminal differentiation (Table 1).

2.3.5. Neuronal/glial differentiation (Table 1)

Although the formation of neural tissue types from spontaneously differentiating PSCs has long been considered a non-specialized default pathway, the promotion of differentiation towards one neural lineage subtype or the other may be manipulated through varied means. While a multiple of terminal neuronal differentiation methods have been documented (see Table 1 and references therein), a few common denominators among them exist. The NSCs, either as rosettes and/or neural tubes or in suspension as neurospheres, are plated on an adhesive substrate, typically laminin in combination with PLO or poly-lysine. The NSCs are typically grown in low-serum or serum-free/serum replacement media that is tailored with defined and specific combinations of growth factors, such as bFGF, and/or signaling molecules. Neural differentiation typically occurs over a period of 2–3 weeks after which cells may be evaluated for differentiation using a variety of methods. These typically involve immunocytochemistry, staining for the emergence of neural lineage-specific markers accompanied by the loss of pluripotency markers (see Table 4) and, ultimately, the demonstration of functional activity such as action potentials for neurons.

Retinoic acid (RA) plays important roles in the development, regeneration and maintenance of the nervous system [69]. In particular, RA promotes the induction of caudally fated neuroepithelial cells [124]. Adherent cultures of PSCs grown in serum-free media supplemented with RA, in combination with bFGF, have been shown to give rise to an enriched neural population (>90%) following mechanical isolation and replating of neural tube-like structures [8]. The effectiveness of RA in promoting differentiation is enhanced by the presence of additional signaling molecules, such as sonic hedgehog (SHH). SHH is a critical factor for patterning of the early embryo, particularly the ventral

<table>
<thead>
<tr>
<th>markers for neural differentiation</th>
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<tr>
<td>Cell Type</td>
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<tr>
<td>Pluripotent stem cell [PSCs]</td>
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<td>Neural stem cell (rosettes)</td>
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<td>Neural precursor cell (other)</td>
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<td>Neural crest cell</td>
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<tr>
<td>Mature neural cell</td>
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<td>Dopaminergic neurons</td>
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<td>Cholinergic neurons</td>
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<tr>
<td>Peripheral neurons</td>
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<td>Sensory neurons</td>
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<tr>
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<tr>
<td>Glial cells</td>
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<td>Astrocytes</td>
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<td>Oligodendrocytes</td>
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neural tube. Treatment of human PSC-derived neuroectodermal cells with retinoic acid (RA) and sonic hedgehog (SHH) has resulted in the generation of spinal motor neurons [61,64,66]. Remarkably, purmorphamine, a small molecule activator of the SHH pathway, can substitute for SHH and, in association with RA, promote the formation of motor neurons [65].

The SHH signaling pathway is also involved in the specification of dopamine (DA) neural subtypes. Treatment of PSC-derived neuroepithelial cells with SHH in combination with fibroblast growth factor-8 (FGF-8) has been shown to promote differentiation of DA neurons with a forebrain phenotype [121]. However, early exposure to FGF during neuroepithelial specification promotes the formation of midbrain dopaminergic neurons [121]. Thus, the sequence and timing of FGF-8 and SHH exposure is critical in determining the choice of neuronal subtype produced.

Tyrosine hydroxylase-positive (TH+) midbrain dopamine (DA) neurons have also been successfully differentiated from PSCs following co-culture with PA6 stromal cells and directed differentiation with bFGF and AA. Although these PSC-derived DA neurons demonstrated functionality in vitro, they failed to have any beneficial effect following transplantation into the striatum of hemi-Parkinsonian rats [86]. It has become evident that there are many combinations of neural differentiation agents that can produce a similar, marker-defined, output. For example, TH+ DA neurons have also been induced by culture in the presence of noggin and dibutyryl cAMP (dbcAMP) [43]. Transplantation of these neuronal progenitors into the striata of 6-hydroxydopamine treated rats produced functionally differentiated DA cells in vivo 2–3 weeks post-transplantation.

A variety of neural differentiation protocols have utilized the developmental signaling molecule noggin [9,37,108]. Noggin is critical for neural tube fusion during embryogenesis. It is a secreted polypeptide that binds and inactivates members of the transforming growth factor-β (TGF-β) superfamily of signaling proteins. In most culture methods, noggin is thought to act to suppress non-neural differentiation pathways. Noggin enhances the directed differentiation of PSCs into neuroectoderm through its antagonistic action on bone morphogenetic protein-4 (BMP4) signaling pathways [45].

Recently, neural crest stem (NCS) cells have also been derived from PSCs at the neural rosette stage. Neural crest precursors (expressing p75 and HNK1) were isolated from neural rosettes by fluorescence-activated cell sorting (FACS) and expanded in culture using BFGF and EGF. Neural differentiation was induced by the withdrawal of BFGF/EGF and exposure to BDNF, GDNF, NGF, and dbcAMP. This treatment regimen yielded both peripheral sympathetic neurons (TH+/Peripherin+) and sensory neurons (Brn3a+/Peripherin+). Schwann cell differentiation was also induced from these expanded p75+/HNK1+ cultures by treatment withCNTF, neuregulin 1 β, and dbcAMP. Critically, transplantation of the human NCS cells into the developing chick embryo and the adult mouse demonstrated survival, migration and differentiation compatible with neural crest identity [60].

The directed differentiation of PSCs towards oligodendrocytes has also been achieved. In one method, oligodendrocyte progenitor cells (OPCs) have been produced from EBs cultured with a combination of bFGF, EGF and RA [78]. These OPCs are reported to be capable of functional integration and compact myelin formation following transplantation into the shiverer mouse model of dysmyelination [78]. OPCs have also been differentiated from mechanically isolated rosettes and neural tubes that were cultured with a regimen of bFGF and EGF for four days followed by N2, bFGF and PDGF for an additional 8 days, prior to growth factor withdrawal and treatment with the thyroid hormone 3,3′,5-triiodo-t-thyronine[50]. Using this method, over 80% of the total cell population were oligodendrocytes. Importantly, these OPCs, when co-cultured with rat hippocamal neurons, were capable of myelinating axons.

Astrocytes are the most abundant cell type in the brain and are critical for the normal physiology of the CNS. Although methods for the directed differentiation of PSCs towards the astrocyte lineage remain scant, neural progenitors derived from PSCs, however, do generate astrocytes during gliogenic stages in the absence of any defined treatment [115]. The generation of astrocytes is still poorly understood. The identification of specific markers for the identification of astrocyte precursors is a necessary first step. Indeed, the molecular pathways involved in the differentiation of many specialized neural subtypes remain to be fully unraveled.

2.4. Comparison of heNSCs and hbNSCs (Fig. 6)

Neural stem cells which were derived from PSCs or from somatic tissues most likely represent two (or even a large number of) phenotypes with different biological properties (Fig. 6) [2,27,33,77,103,106]. The only commonalities among them are the expression of certain markers (Table 4) and neuronal-like functional properties when differentiated in vitro or in vivo [62]. Not only do these two cell types display very different expression patterns, even when looking at “marker” genes, they may vary considerably in the neuronal phenotypes which evolve after differentiation in vitro or after transplantation in vivo [2,27,33,106]. Currently, there is considerable discordance between what we assume about the identity and fate of neural stem cells (from any source) and experimental, especially quantitative, insight into what each possible stem cell type actually does mature into when subjected to differentiating conditions.

What we now know is that neural progenitors and stem cells derived from somatic sources or PSCs can form neuronal connections and participate in functional neuronal circuits [62,75,125]. We do not know for sure how physio-
The lack of such an assay is mostly due to the fact that there is no experimental method, such as the repopulation experiments in the hematopoietic stem cell field, which can unequivocally demonstrate that a single cell or cell preparation does indeed have the ability to differentiate and assume the function of every cell type within a specific organ system. One major reason for this is that the neurological equivalent of the hematopoietic experiments raises some profound ethical issues [48,104].

At present, only the neural rosette cell can currently be regarded as the “true” neural stem cell [33]. Neural stem cells from somatic sources are considerably different from neural stem cells from ePSCs (Fig. 6) [2,27,33,106]. Every neural progenitor and stem cell type that has been derived, even just from slightly different sources (age of subject, marker based sorting, ePSC-line of origin, brain region, culture method), may possibly represent its own neural stem cell phenotype with unique properties within a wide spectrum of possible functions. Only an unbiased, scientific phenotyping examination of every reported neural stem cell preparation using systematic, high-throughput, functional genomics, combined with in vitro and in vivo assays, will be able to tell us if any generalizations can be made about what we now call “neural stem cells”.

2.5 Concluding remarks

2.5.1 Differentiation ability of different ePSC lines

One issue that is particularly important is whether all PSC lines have equal capacity to differentiate into a particular cell type. There have been many anecdotal reports of cell lines that are better than others at producing neurons, or glia, or pancreatic islet cells, but there have been only a few publications on the multiple cell line comparisons that would be necessary to demonstrate differences in potency. In one study [56], HSF6, Miz-hES4, and Miz-hES6 were compared for their ability to give rise to cells of ectodermal, mesodermal, and endodermal lineages following EB formation and subsequent plating. The data convincingly show that there were marked differences in differentiation potential among the three lines. In the second study [79], HUES lines 1–17 were examined with a finding similar to the previous study: some lines showed a marked propensity to differentiate into specific lineages. Importantly, even within a given line, the variability of specific lineage differentiation appears to be quite large.

Although no specific causes for the variability in differentiation potential have yet been found, the differences are most likely due to genetic variations, epigenetic modifications [3,13,55], or both. There is abundant evidence that PSC lines accumulate genetic alterations during culture [3]. These may be gross aneuploidies or subtle differences that can be detected only by high-resolution genetic mapping techniques such as SNP genotyping. Genetic changes could affect the levels of expression of genes, either directly
through mutations in protein-coding sequences, or indirectly through affecting regulatory elements such as transcription factors and miRNAs. Epigenetic factors, such as DNA methylation and chromatin modifications are known to change during cellular differentiation, but it is unclear which changes are causal and which are the result of the differentiation. Both genetic and epigenetic changes could occur very early in the derivation of PSCs, and influence their future direction. Such changes would not be detectable by currently available analysis methods. Since most culture conditions are developed with the goal of preventing PSCs from differentiating, early events that predetermine their later differentiation would not be noticed.

2.5.2. Homogeneity of final product
Production of a “pure” population of differentiated cells may, in fact, be possible in vitro. What remains, however, is to determine whether or not a homogeneous final product is actually desirable. This will depend entirely on its application. It is probable that a homogeneous final product will not be best for most applications. In the transplantation arena, for example, although a pure population of oligodendrocyte progenitors may be desirable for the demyelination subsequent to spinal cord injury, this form of therapy alone does not address the many other consequences of spinal cord injury on neuronal populations. Indeed, in most forms of nervous system injury more than one type of cell is affected, not only glia and neurons, but different types of neurons, both intrinsic and extrinsic. A related consideration can be made for in vitro studies, whether they be aimed at understanding neurobiology in health or disease or whether they are designed to screen for small, therapeutic, or reagent molecules. That is, no part of the nervous system is comprised of a single cell type; thus, multiple cell types are not only desired, but required, to recapitulate normal nervous function in vitro. At present, only relatively passive methods for directing an appropriate multi-lineage differentiation sequence exist and these depend entirely on the abilities of the resulting cells to self-assemble into semi-functional neural nets.

2.5.3. Expandability of final product
Generation of sufficient quantities of appropriately differentiated cells will drive the methodology. That is, whether or not any of the progenitors to the cells desired, besides the PSCs themselves, can be expanded in vitro will determine which step in the differentiation process will also be the expansion step. Other considerations include ease and cost of expansion and untoward effects of expansion such as genetic instability. For example, while it seems to be relatively straightforward to expand neural progenitors of limited differentiation potential (i.e. stage 4 cells) in vitro, expansion of neural rosette cells with more differentiation potential (i.e. stage 3 cells) appears to be more difficult. This may be due to a requirement of these cells for a particular two- (or three-)dimensional arrangement with particularly close cell-to-cell contacts. Generation of large quantities of these cells, therefore, may require a large quantity of their immediate precursors, EBs, and, by extension, PSCs. Expansion of PSCs, however, can give rise to aneuploidies; thus, additional care must be taken in characterizing the starting material before a large scale differentiation protocol is instituted.

2.5.4. Tumorigenicity of final product
One of the basic properties of undifferentiated PSCs is their ability to form a certain type of tumor called a teratoma, which is a tumor comprised of disorganized foci of differentiated tissues of different lineages. The differentiation potential of PSC lines is typically determined by injecting a bolus of undifferentiated PSCs into the muscle of a SCID mouse and then evaluating the resulting tumor some weeks later. So far, all ePSC lines have demonstrated the ability to form teratomas. The implantation of undifferentiated PSCs, therefore, carries substantial risk [5]. Undifferentiated PSCs are thus unlikely to be used directly in therapeutic applications. Differentiation of a PSC, however, is thought to eliminate its capacity to form a tumor. Therefore, PSCs will be used to generate populations of cells that are at least partially differentiated, and these differentiated cells will be used therapeutically [42]. Two possible confounds to this general principle, however, have recently been described in the literature: (1) the continued in vivo proliferation of neural cells differentiated from PSCs [33,98] and (2) the production of a much more malignant tumor when PSCs were implanted into human, rather than mouse, tissue [105]. Thus it is not only unclear which differentiated progeny of PSCs are appropriate for transplantation but also how, exactly, to even determine human tumorigenicity of these differentiated populations.

Acknowledgments
We gratefully acknowledge Boback Ziaeian for performing the immunocytochemistry and generating the fluorescence photomicrographs and Hubert Nethercott, Michele Sheridan, and Heather Maxwell for performing ePSC culture. We also thank Robin Wesselschmidt for critically reading the manuscript. The work in the communicating author’s laboratory is supported by the CHOC Foundation for Children and the National Institutes for Health.

References