Cytotherapy, 2013; 15: 999–1010

International Society for Cellular Therapy
ISCT®

Optimizing dopaminergic differentiation of pluripotent stem cells for the manufacture of dopaminergic neurons for transplantation

QIUYUE LIU[1], OLIVER Z. PEDERSEN[1], JUN PENG[1], LARRY A. COUTURE[2], MAHENDRA S. RAO[3] & XIANMIN ZENG[1]

[1]*Buck Institute for Age Research, Novato, California, USA,* [2]*Bechman Research Institute, City of Hope, California, USA, and* [3]*National Center of Regenerative Medicine, National Institutes of Health, Bethesda, Maryland, USA*

Abstract

Background aims. We have previously described a xeno-free scalable system to generate transplantable dopaminergic neurons from human pluripotent stem cells. However, several important questions remain to be answered about our cell therapy efforts. These include determining the exact time at which cells should be transplanted and whether cells at this stage can be frozen, shipped, thawed and injected without compromising their ability to mature and survive the transplantation procedure. We also needed to determine whether further optimization of the culture process could shorten the development time and reduce variability and whether a current Good Manufacture Practice (CGMP) facility could manufacture cells with fidelity. *Methods.* We developed an optimized protocol that included modulating the sonic hedgehog homolog gradient with bone morphogenetic proteins (BMP2) and addition of activin to the culture medium, which shortened the time to generate Lmx1A and FoxA2 immunoreactive cells by 4–6 days. *Results.* We showed that cells at this stage could be safely frozen and thawed while retaining an excellent ability to continue to mature *in vitro* and survive transplant *in vivo*. Importantly, we successfully adapted this process to a CGMP facility and manufactured two lots of transplant-ready dopaminergic neurons (>250 vials) under CGMP-compatible conditions. *In vitro* characterization, including viability/recovery on thawing, whole genome expression as well as expression of midbrain/dopaminergic markers, showed that the cells manufactured under GMP-compatible conditions were similar to cells produced at lab scale. *Conclusions.* Our results suggest that this optimized protocol can be used to generate dopaminergic neurons for Investigational New Drug enabling studies.

Key Words: *cell therapy, dopaminergic neuron, GMP, human pluripotent stem cells*

Introduction

Cell-based therapy and combination of cells with genetic engineering strategies are being considered for the treatment of Parkinson disease when other options are not available. These cell-based therapeutic approaches are built on decades of experience in treating patients with cells sourced from fetal tissue (1–4). The collective experience has shown that whereas cell-based therapy is safe and useful in at least a subset of patients, there are several issues that must be resolved. These include dyskinesias, variable efficacy of the transplant, the possibility that transplanted cells will be affected by the ongoing disease process, the limited availability of matched fetal cells for transplant and the possible negative effect of immune modulators on cell survival (5).

To address some of these concerns, investigators have turned to identifying alternate sources of dopaminergic neurons. Human pluripotent stem cell (PSC)-derived dopaminergic neurons offer some potential advantages. For example, cells can be obtained in large numbers, selection processes are available if purified cells are necessary and in theory, autologous cells with the use of an induced pluripotent stem cell (iPSC)-based strategy or a cell banking strategy can provide matched cells that will not require immunosuppressive therapy.

Indeed, the possibility of a successful therapeutic intervention has led several groups, including our own, to develop protocols for the generation of functional dopaminergic neurons from both embryonic stem cells (ESC) and iPSC. Several publications that used laboratory-scale processes with defined culture conditions have shown that in principle it is possible to scale up processes to obtain therapeutically relevant numbers of cells (6,7). The process that we have described allows for banking of cells at a stable intermediate neural stem cell (NSC) stage. This allows the overall 40-plus day differentiation process to be managed more effectively at a manufacturing

Correspondence: **Xianmin Zeng**, PhD, Buck Institute for Research on Aging, 8001 Redwood Blvd., Novato, CA 94945, USA. E-mail: xzeng@buckinstitute.org

(Received 26 December 2012; accepted 2 March 2013)

http://dx.doi.org/10.1016/j.jcyt.2013.03.006

site. Cells produced with the use of this staged protocol show no contamination with undifferentiated PSC, do not form ectopic masses when transplanted (6,7) and are otherwise similar to preparations using other procedures (8).

More recently, Studer *et al.* reported additional determinants of the quality of midbrain dopaminergic neurons (9). Although their process of dopaminergic differentiation is largely similar to the one that we have described, their protocol differs in the timing of exposure to the common cytokines used. Their data suggest that expression of Lmx1a and FoxA2 is important in the development of the A9/A10 midbrain dopaminergic neuronal phenotype and that many of the culture processes used earlier did not determine this type of the midbrain neuronal phenotype.

Another important unanswered question is the exact time at which cells should be transplanted and what level of purity is required (10). It is estimated that, at least with fetal cultures, no new cells are born after transplant, and the number of surviving dopaminergic cells in a transplant represent the number of available for functional replacement (1,4). Because survival of transplanted cells is in inverse correlation to their maturity, this suggests a limited time window to transplanted cells (11). Similarly, whereas ancillary cells, astrocytes in particular, are important for the survival and integration of dopaminergic cells (12,13), we are also constrained by the absolute number of cells that can be delivered to a particular site and the density of a cell suspension that can be delivered through a fine-gauge needle without compromising the health of the transplanted cells (6).

In an attempt to address these issues, we examined our differentiation protocol and modified it to shorten the time to maturation and increase the percentage of Lmx1A/FoxA2-expressing dopaminergic neurons. We determined an optimal stage at which cells could be safely frozen, shipped and thawed without compromising their ability to continue to mature *in vitro* as well as to survive after transplantation in rodents. We show that modulating the sonic hedgehog homolog (Shh) gradient with bone morphogenetic proteins (BMP2) and adding activin to the culture medium alters the final composition significantly. These conditions produced large numbers of Lmx1a- and FoxA2-immunoreactive cells that then matured to form dopaminergic neurons that release dopamine in culture. Under these modified conditions, cells can be safely frozen at day 14 of the culture period while retaining excellent survival and ability to continue to mature *in vitro* as well as to survive after transplantation in rodents *in vivo*. Most importantly, we manufactured cells at a scale suitable for preclinical and clinical studies with the use of a current Good Manufacture Practice (CGMP)-compatible process and tested the equivalency of such cells to those generated by means of a laboratory-scale process. Our results from two production runs in the GMP facility confirm that lots manufactured under CGMP-compatible conditions are consistent from lot to lot and similar to cells produced in the laboratory.

Methods

Dopaminergic differentiation of NSC

NSC were derived from ESC and maintained on culture dishes coated with Geltrex (Invitrogen; Carlsbad, CA, USA) as previously described. The following two protocols were used for dopaminergic neuron differentiation: (i) NSC were plated on poly-ornithine-laminin–coated culture dishes in STEMPRO medium (Dulbecco's modified Eagle medium/F-12 + GLUTAMAXTM medium with STEMPRO human ESC [hESC] serum- and feeder-free medium growth supplement, 1.8% bovine serum albumin [BSA] and 8 ng/mL fibroblast growth factor [FGF]2) supplemented with SHH (200 ng/mL) and FGF8 (100 ng/mL). Ten days after differentiation, cells were differentiated in STEMPRO medium supplemented with brain-derived neurotrophic factor (BDNF) (20 ng/mL), glial cell line-derived neurotrophic factor (GDNF) (20 ng/mL) and dibutyryl cyclic AMP (dcAMP) (0.1 mmol/L). After 5 days of differentiation, dcAMP was withdrawn and the cells were further differentiated for 1–2 more weeks with a medium change every other day. Cells were split when necessary before 15 days after differentiation. (ii) NSC were plated on poly-ornithine-laminin–coated culture dishes in STEMPRO medium supplemented with Shh (200 ng/mL), BMP22 (4 ng/mL) and activin A (10 ng/mL). Ten days after differentiation, cells were differentiated in STEMPRO medium supplemented with BDNF (20 ng/mL), GDNF (20 ng/mL), dcAMP (0.1 mmol/L) and activin A (10 ng/mL). After 5 days of differentiation, dcAMP and activin were withdrawn and the cells were further differentiated for up to 10 days with a medium change every other day. Cells were split when necessary before 14 days after differentiation.

Cell viability assay

Cell viability was determined by Trypan blue exclusion test. In brief, cells suspension was mixed with equal volumes of 0.4% Trypan blue solution and applied onto a hemocytometer chamber, C-Chip DHC-N01 (INCyto) according to the manufacturer's instruction. Viable cells were not stained, whereas dead cells were stained blue.