In Vitro And In Vivo Characterization Of Clinical Grade Human Undifferentiated Neural Progenitor Stem Cells (Npc) For Treatment Of Neurodegenerative Diseases

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Abstract

Pre-differentiated stem cells of various origins are being tried for the treatment of neurodegenerative disorders. Pre-differentiation has two flaws: first, it is difficult if possible to determine the correct dose of the specialized neural cells for an individual patient and, second, the specialized cells can address only single factor of usually multifactorial neurodegenerative disorders. To address these problems, a suspension close system bioreactor was used to manufacture a line of NPCs that are able to tune their proliferation and vector of differentiation in response to micro-environmental cues. Immunofluorescence, flow cytometry, and teratoma formation assays were used to demonstrate that NPCs retained pluripotency, potency, genetic stability and sterility of the cell product. The NPCs express high levels of Oct-4, Sox-2 and low levels of MHC-I. Proteomic analysis revealed that the cells secrete a number of proteins that have neuroprotective qualities such as - Neuromodulin, 14-3-3 epsilon protein, Ceruloplasmin, Insulin Degrading enzyme, Insulin like Growth Factor II, and Activity dependent neuroprotective protein. Stereotactic injection of the NPCs into the cerebellum of spastic Han-Wistar (sHW) rat model of cerebellar ataxia, lead to repopulation of the depleted Purkinje cell layer with Calbindin-positive NPCs, and nearly complete amelioration of forelimb tremor, hind-leg rigidity, and gait abnormality, characteristic for sHW rats. Stereological analysis demonstrated significant increase in the number of the rat Purkinje neurons as compared to control animals. These data justify consideration of NPCs for development of regenerative treatments for neurological disorders.

Introduction

PD, cerebellar ataxias and other neurodegenerative diseases have multifactorial pathogenesis, where both genetic (Ibanez et al., 2017; Lill and Klein, 2017) and non-genetic environmental factors (Cannon and Greenamyre, 2011) are involved.
Current pharmacological and biological approaches to the treatment of Parkinson's disease (PD) attempt to alleviate the disease through dopamine (DA) replacement. The most common investigative approach to stem cell (SC) treatment of PD is to use SCs that are pre-differentiated into DA-producing neurons before implantation into the brain (Armstrong et al., 2001); (Fitzpatrick et al., 2009). However, DA deficiency is only one of multiple factors that contribute to the pathology of PD. This strategy addresses the dopamine deficiency only and raises a difficult question of how many dopamine producing cells to inject into an individual patient, taking into account that there are no two patients alike. Another negative aspect of using pre-differentiated cells is that they are, in effect, a DA replacement therapy. Thus, just like levodopa, pre-differentiated cells cannot address non-motor components of the disease that are non-dopaminergic in origin. And just like levodopa, they can never be curative (Barker et al., 2013). Undifferentiated stem cells are, by definition, more plastic than pre-differentiated ones. It is hypothesized that they are able to adjust their rate of proliferation and path of differentiation according to micro-environmental cues they read from the host brain in the same manner as occurs during normal development of a fetus. These qualities of undifferentiated cells may allow them to address dopaminergic and non-dopaminergic aspects of the disease, as well as to avoid mis-dosing the patients. We believe human allogeneic neural stem cells (HANSCs) we are using retain the plasticity and the advantages of embryonic cells, but lack their tumorigenicity.
Working hypothesis: HANSCs can be safely implanted in the PD brain, and then follow microenvironmental cues, multiply according to the severity of the brain damage, and differentiate into different types of brain cells according to the nature of the damage.

Materials and Methods

CELL CULTURE
Human fetal brain tissue was procured via routine sterile manual aspiration. Maternal blood samples (sera) were tested for: HIV, hepatitis A, B and C; HTLVI; VDRL; and cytomegalovirus. Fetal brain was dissected, minced and triturated to a single cell suspension. Cell suspension samples were cultured under hypoxic conditions in ultra-low attachment, feeder free flasks with xeno free Eagle's essential medium (Hyclone, SH30310, Logan UT, USA), supplemented with Gem21 (Gemini Bio-Products, 400-660, Sacramento, CA, USA), and a number of other trophic factors all of which were added at proprietary concentrations.

Materials and Methods

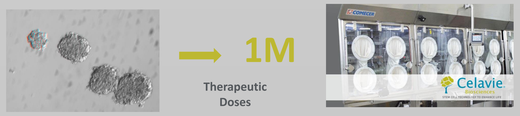
At the second doubling (D2) cell culture was tested for sterility (USP <71>); at D4 - karyotyped and PCR tested for adventitious agents required by the Donor Screening Protocol. Cells were then transferred to a closed bioreactor system (GE WAVE Bioreactor 2/10 System, Uppsala SWE).At D7 the Master Cell Bank (MCB) was harvested, safety tested, characterized and rate-control cryopreserved. A portion of the batch was thawed and used to seed the bioreactor to produce the Working Cell Bank (WCB) at D13. The WCB was subjected to release testing for safety and characterization assays. Safety testing included sterility (USP <71>), mycoplasma (USP <63), endotoxin (USP <85>), and karyotyping. Characterization included flow cytometry testing for: Oct-4 >90%; Sox-2 >90%; MHC-I <10%; MHC-II <10%; CD105 <10%; PE-Cy7 conjugated), and tyrosine hydroxylase <10%. Both MCB and WCB were stored in gas phase LN2.
All procedures were performed under aseptic conditions in an ISO 8 clean room, utilizing ISO 5 bio-safety cabinets and laminar airflow hoods, according to validated protocols.
IMMUNOFLUORESCENCE
After standard preparation of the cytocentrifuged slides, Oct4, Sox2, and MHCI primary antibody dilutions were applied to the slides and incubated for 24 hours at 4°C. Then, slides were washed and incubated with Alexa Fluor 594 secondary antibody in the dark for 1 hour at room temperature. Slides were washed in IHC washing solution, coverslipped, sealed, an imaged with Toup View 6.3 software.
FLOW CYTOMETRY
All antigen-labeled samples were centrifuged at 1000rpm for 5 minutes and run in triplicates. Cell pellets were resuspended in 1X PBS with MHC-I antibody. Samples were vortexed and incubated for 15 minutes in the dark, fixed in Reagent A for 15 minutes, washed in 1X PBS, centrifuged at 1000rpm for 5 minutes and resuspended in 100ul Reagent B containing Sox2 and Oct4 antibodies, incubated for 20 minutes, washed, centrifuged and resuspended in 500ul 1X PBS for Flow Analysis. All samples were analyzed with the Attune focusing cytometer (Blue/Red, Life Technologies) using BL1 (Sox2), BL2 (Oct4), and RL1 (MHC-I) fluorescence channels.
Proteomic analysis
Sampes of WCB were analyzed for protein expression by National Research Center of Canada

Animals: Spastic Han Wistar rats (sHW) were obtained from California State University, Northridge's breeding colony. The spastic Han Wistar rat represent a model for human ataxia characterized by forelimb tremor, hind-leg rigidity, gait abnormality, weight loss, shortened life span (about 65 days), caused by neurodegeneration of cerebellar Purkinje cells and hippocampal CA3 pyramidal cells (Cohen, 1991, 1997). An equal mix of male and female rats were divided into four treatments: an untreated normal group (n=10), an untreated, mutant group (n=10), a dead Neural Progenitor Cell (NPC) treated, mutant group (n=9), and a live NPC-treated, mutant group (n=10).
NPC TREATMENT:
Starting at 30 days of age, sHW rats were implanted with Alzet osmotic pump (Model 2004; 28 day duration) to infuse chronically cyclosporine (15 mg/kg/day). Ten days later, they received bilateral injections of 500,000 NPCs (1,000,000 cells total) or dead NPCs into the cerebellum (AP -11.0 mm; ML ±2.0 mm; DV 5.5 mm). Dead NPCs were used to control for any paracrine effects of NPC inoculations and were obtained from those live populations and frozen at -20°C until use.
ROTAROD TESTING:
The rotarod to test balance, grip strength and motor coordination was used to measure cerebellar decline (Uhlendorf, 2017).

Results

Commercial Scale cGMP Production of Clinical Grade Stem Cells

- Industry-accepted, multi-tier cell banking system
- Meets stringent FDA/CVM guidelines for viability, genetic stability and absence of infectious agents
- Proprietary Isolation technology produces highly standardized, genetically stable cell lines, with uniform phenotypic characteristics



Biologically Active Proteins Secreted by the Stem Cells as Detected by Proteomic Analysis:

- Neuromodulin - promotes neuronal growth, plasticity protection
- 14-3-3 Epsilon Protein – reduces neurotoxicity induced cell death.
- Insulin Like Growth Factor II - Neuroprotective effect in aging brain
- Activity Dependent Neuroprotective Protein - Down-regulation contribute to dopaminergic neurodegeneration in PD
- Ceruloplasmin - Injections were shown to reverse cell death in substantia nigra (PD).

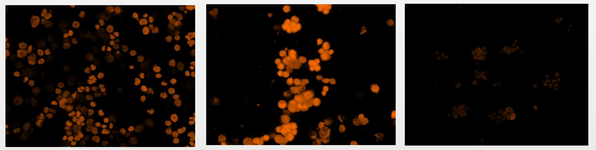


Figure 1. Immunofluoresence analysis: A, Sox-2; B, Oct-4; C, MHC-I expression in NPCs

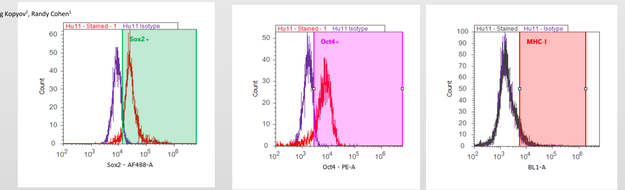


Figure 2. Flow Cytometry analysis: NPCs are 94% positive for Sox-2, 89% for Oct-4, 8% for MHC-I

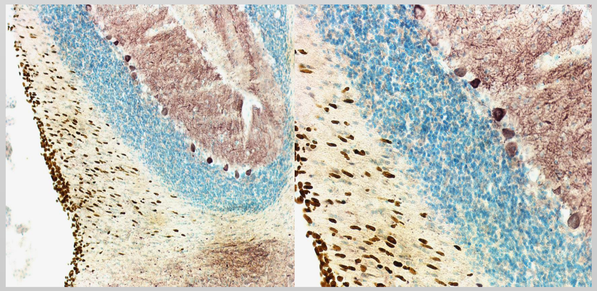


Figure 3. Immunohistochemical staining of the cerebellum of a mutant sHW rat that received injection of live human stem cells show human nuclei-labeled stem cells migrating from the needle tract towards the granule cell layer. Note the double-labeling of some human stem cells with Calbindin. Human Nuclei (brown) at 1:200 dilution. Calbindin (purple) at 1:200 dilution. Methyl Green counterstain (blue). 100X and 200X, respectively.

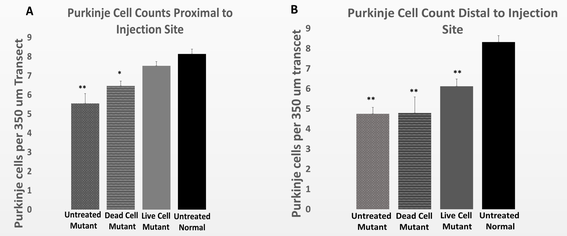


Figure 4. Purkinje cell (PC) counts of each group. (**A)** Cell counts proximal to the injection site and anatomically similar areas in rats with no injections. Live NPC-treated mutants (n=10), dead NPC-treated mutants (n=9), mutant untreated rats (n=10) and untreated normal rats (n=10) were all counted. Analysis revealed statistical differences across all treatments (F=12.39, p<0.05). The live NPC-treated mutants showed a statistically significant larger number of PCs than in dead NPC mutants (p<0.05) and mutant untreated rats (p<0.001) and no statistical difference with the normal untreated rats (p>0.05). (**B)** Cell counts distal to the injection site (>1 mm) and similar areas in rats with no injections revealed statistical difference across all treatments (F=13.394, p<0.001. Data shown are mean±SEM, one asterisk indicating significant differences in PCs between live NPC mutants and dead NPC mutants; double asterisks indicating significant difference in Purkinje cells between live NPC mutants and untreated mutant rats (ANOVA; Tukey's Post-Hoc Test).

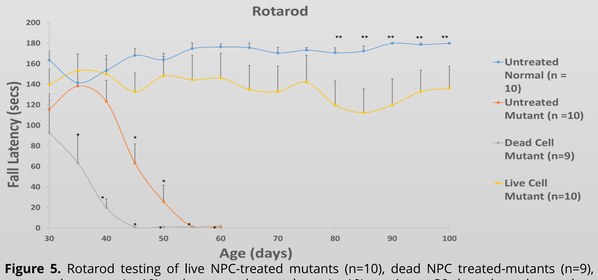


Figure 5. Rotarod testing of live NPC-treated mutants (n=10), dead NPC treated-mutants (n=9), untreated mutants (n=10) and untreated normal rats (n=10) starting at 30 days shows better than control rats performance and extended life span comparable with normal rats. Statistical significant was found across treatments (F=25.03, p<0.001). Data shown are mean ± SEM; single asterisks indicate significant difference in fall latency between live NPC and dead NPC mutants or non-treatment mutants, and two asterisks indicate differences between live NPC mutants and untreated normal rats (Repeated Measures ANOVA; Tukey's Post-Hoc Test).

Conclusion

Celavie technological platform reproducibly manufacturers genetically stable homogenous human stem cell lines with standardized phenotypic, proteomic and morphological characteristics.
Celavie allogeneic undifferentiated pluripotent stem cells can migrate to the site of damage and differentiate into Purkinje-like cells corresponding to specific microenvironment cues. They secrete biologically active neuroprotective and anti-inflammatory proteins thereby stimulating the host's own protective and regenerative pathways, which is reflected in the increase in the numbers of the rats' own Purkinje cells. Such structural repair resulted in restoring behavioral parameters and extending the life span of the mutant rats.