Otic Neuronal Differentiation of Three-Dimensional Spheroids Derived from Human Pluripotent Stem Cells: Characterization Towards In Vivo Transplantation

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Background

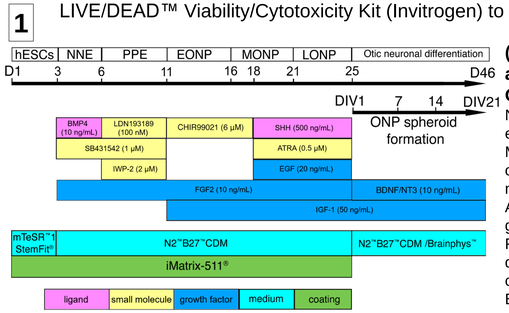
Although inner ear stem cell replacement therapies are a promising potential method to support regeneration of spiral ganglion neurons, transplanted cells must be characterized to ensure they maintain the proper otic neuronal progenitor (ONP) characteristics and a system must be developed for transplant to the inner ear with minimum stress on the cells. Here, we develop three-dimensional (3D) ONP spheroids for transplant and establish a micropipette-based transfer system to efficiently transplant ONP spheroids to the inner ear at a suitable stage of development.

Research Objectives

- Develop human embryonic stem cell (hESC) derived ONP spheroids and ensure cell survival as well as expression of ONP characteristics
- Determine optimum point of ONP spheroid development for transfer to the inner ear
- Create a micropipette spheroid transfer method to transplant spheroids efficiently while maintaining 3D structure
- Analyze transplanted spheroids to ensure maximum cell survival

Methods

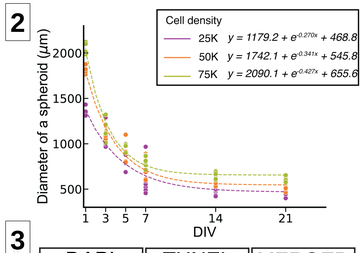
1. Generation of ONP spheroids: differentiation has been previously described [1]. Briefly, hESCs (H1, H7, H9, Wicell) were treated according to the diagram below. At day in vitro (DIV) 1, cells were seeded to 3D spheroids using a 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (Corning Life Science) or an EZphere® culture plate (Nacalai).
2. Characterization of ONP spheroids: fixed spheroids were analyzed by terminal deoxynucleotidyl transferase dUTP nick-end-labeling (TUNEL) staining and immunocytochemistry. Live spheroids were examined by patch clamp to measure electrophysiological properties and nanoindentation to measure Young's modulus using a Piuma nanoindenter (Optics11).
3. Micropipette mock transplant of ONP spheroids: transplantation was simulated using a Xenoworks Digital Injector (Sutter Instrument) system with micropipettes in a range of sizes. For transfer, negative pressure was manipulated to hold the spheroid in place at the pipette tip. After transfer to the desired area, positive pressure released the spheroid from the micropipette. Data were analyzed to describe a relationship between the pipette/spheroid size ratio and the negative and positive pressures required for transfer.
4. Analysis of transferred spheroids: post-mock transplant, spheroids were examined with a LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen) to track patterns of cell death.

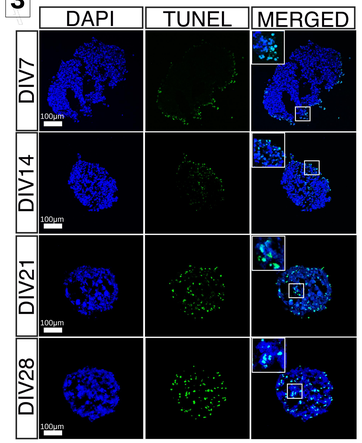


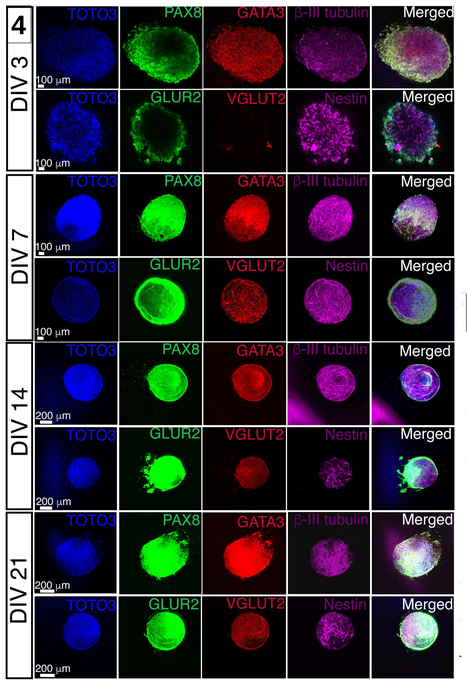
(1) Schematic summary of the protocol and timeline for generating late-stage ONPs from undifferentiated hESCs. NNE: nonneuronal ectoderm; PPE: preplacodal ectoderm; EONP: early otic neuronal progenitor; MONP: mid-otic neuronal progenitor; LONP: late otic neuronal progenitor; BMP4: bone morphogenetic protein 4; SHH: Sonic hedgehog; ATRA: all-trans retinoic acid; EGF: epidermal growth factor; IGF-1: insulin-like growth factor 1; FGF2: fibroblast growth factor 2; BDNF: brain derived neurotrophic factor; N2B27-CDM: chemically defined medium containing N2 and B27 supplements.

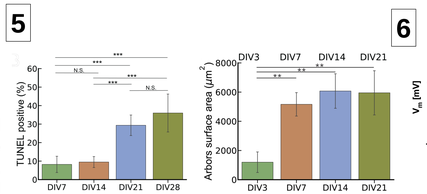
1. Matsuoka AJ, Morrissey ZD, Zhang C, Homma K, Belmadani A, Miller CA, et al. Directed differentiation of human embryonic stem cells toward placode-derived spiral ganglion-like sensory neurons. Stem Cells Transl Med. 2017 Mar;6(3):923–36.

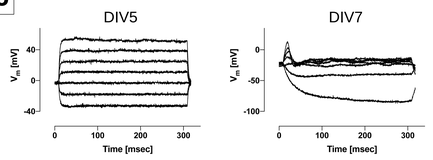
Results









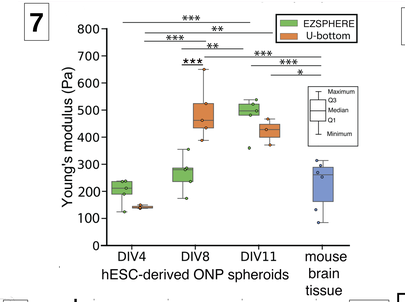


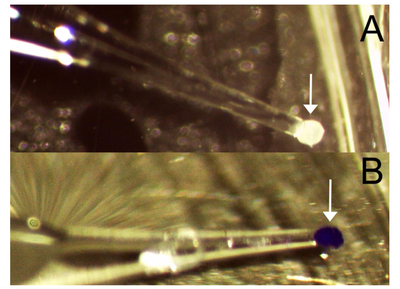
Characterization of hESC-derived ONP spheroids, pre-transplant. (2) Curve fit showing spheroid diameter change over culture period. (3) TUNEL staining of center sections of spheroids at weekly time points. TUNEL-labeled cells show the DNA breaks indicative of cell death. (4) Immunocytochemical staining of spheroids for ONP markers. (5) Quantification of TUNEL staining (left) and neurite arborization (right) of spheroids. Error bar: standard error. N.S.: not significant. Asterisks indicate statistical significance by ANOVA with Tukey post hoc test ($p < 0.001$). (6) Whole-cell current-clamp recording of ONP spheroids at DIV5 (left) and DIV7 (right) showing transmembrane potential (Vm) repolarization after current-induced depolarization in DIV7 spheroids.

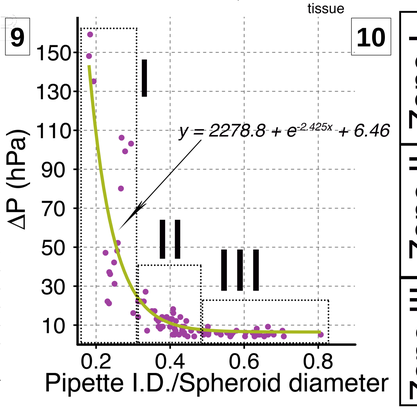
Disclosure

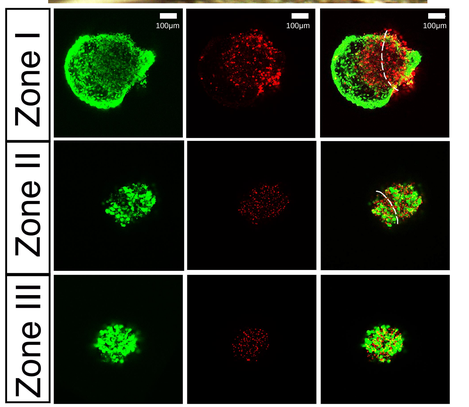
There are no financial conflicts of interest to disclose. Any kind of financial support (funding, grants, sponsorship) received has been acknowledged (see right column)

Results (Continued)









Mock transplant of hESC-derived ONP spheroids. (7) Young's modulus measurements of spheroids formed by 96-well (U-bottom) plate and EZsphere®. Spheroids with a stiffness of approximately 400Pa or greater were robust enough to hold up to micropipette transfer. *, **, and *** indicate statistical significance with two-way ANOVA with Tukey post hoc test ($p < 0.05$, $p < 0.01$, and $p < 0.001$). (8) Micropipette apparatus with spheroids (white arrows) held by negative pressure. (9) Pressure changes required to capture and release individual spheroids, plotted against the ratio of micropipette inner diameter (I.D.) to spheroid diameter. Green line indicates nonlinear curve fit. (10) Representative images of post-transfer spheroids in three zones indicated in (9). Green: live cells, red: dead cells. White dotted line indicates location of pipette contact. Spheroids with a smaller pipette/spheroid size ratio showed greater cell death around the point of contact.

Conclusions

Our hESC-derived ONP spheroids are a promising candidate for transplant to the inner ear in therapies aimed at aiding the regeneration of spiral ganglion neurons. After seven days of in vitro culture, spheroids express appropriate neuronal progenitor markers as well as transmembrane potential repolarization (a preliminary characteristic of all types of neurons). Additionally, cell death is relatively low at this point. Our micropipette spheroid transplant system transfers spheroids efficiently, maintaining 3D form; micropipettes closer in size to the spheroids themselves require less pressure and cause less stress and deformation on the spheroids. To our knowledge, this is the first study to explore the transplantation of cell spheroids using a micropipette and digital microinjector, and thus represents a bridge between in vitro experiments and in vivo transplantation studies.

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