

AWARD NUMBER: W81XWH-18-1-0752

TITLE: Stem Cell Regeneration of Human Spiral Ganglion Neurons Toward Hearing Restoration

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14. ABSTRACT: Hearing loss is a major clinical complaint of military personnel exposed to loud noise known to damage cochlear hair cells and auditory neurons. Our long-term goal is to mitigate this damage through "next-generation" cochlear implants (CIs) that incorporate transplanted human stem cells guided to become new, functional auditory neurons. By combining clinically proven CI methodologies with developing stem-cell-based neural replacement strategies, we seek to achieve large CI performance gains by establishing a new intracochlear neural network to improve CI-neuron connectivity, using the CI to stimulate this network. Our results in Aim 1 allowed us to develop effective means of transplanting stem-cell-derived spiral ganglion neurons (SGNs) into the scala tympani. Our comparisons between a volume flow system and a pressure regulation system for transferring stem cell-derived otic neuronal progenitors (ONP) spheroids and organoids demonstrated that a pressure regulation system resulted in fewer cell death, milder deformities. Our significant findings indicate that hESC-derived 3-D aggregates maintained the molecular and functional characteristics similar to those of otic ONP cells, which are upstream in the SGN lineage. Our results in aim 2 demonstrated that the transplanted stem cell-derived ONP spheroids survived and neuronally differentiated into otic neuronal lineages in vivo. They extended neurites toward the cochlea's bony wall 90 days after the transplantation without the use of immunosuppressant medication.					
15. SUBJECT TERMS hearing restoration, stem cell regeneration, regenerative engineering, regenerative medicine, material science					
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1. INTRODUCTION:

The Veterans Administration reports that hearing loss is among the top medical concerns of service personnel. Our proposed work addresses key roadblocks in the way of translating our “Biohybrid Cochlear Implant (CI)” design to new users. We will evaluate three solutions for improving stem-cell survival and synaptogenesis to the surviving spiral ganglion neuron population, which will benefit all stem-cell-based remedies, regardless of implementing the biohybrid CI concept.

2. KEYWORDS:

hearing restoration, stem cell regeneration, regenerative engineering, regenerative medicine, material science, spiral ganglion neurons, cochlear implant, biohybrid implant, hydrogel

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Optimize the delivery of hPSC-derived multicellular aggregates using a simple *in vitro* model

Major Task 1: Optimize delivery of hPSC-derived 3-D multicellular aggregates using a simple *In vitro* model.

Subtask 1: Achieving Northwestern University IACUC and ACURO approvals (Month 1-3).
Status: completed as of 12/31/2018.

Subtask 2: 3-D multicellular aggregates preparation (generation of inner ear organoids)
Human embryonic cell lines: WA25 (WiCell, WI) (Month 1-2).
Status: completed as of 10/31/2020.

Subtask 3: Three-dimensional multicellular aggregates preparation (generation of auditory neuron spheroids). Human embryonic cell lines: H1, H7, and H9 (WiCell, WI) (Month 1-3).
Status: completed as of 12/31/2018.

Subtask 4 and 6: Evaluate transfer of spheroids/organoids with a volume flow system using Human embryonic cell lines: H1 H7, H9, and WA25 (WiCell, WI) (Month 1-3).
Status: completed as of 12/31/2018.

Subtask 5 and 7: Evaluate transfer of hESC-derived ONP spheroids/organoids using the pressure regulation system using human embryonic cell lines: H1, H7, H9, and WA25 (WiCell, WI) (Month 1-3).

Status: completed as of 10/31/2019.

Subtask 8: Outcome analysis (ANOVA) Possible additional measurements in selective experimental conditions, and writing up results (Month 3-4).

Status: completed as of 10/31/2020.

Specific Aim 2: Create supportive biochemical stem cell niches for successful trans-plantation of hPSC-derived 3-D multicellular aggregates into the cochlea.

Major Task 2: Optimize delivery of hPSC-derived 3-D multicellular aggregates using a simple In vitro model.

Subtask 9: 3-D multicellular aggregates preparation (generation of inner-ear organoids)

Human embryonic cell lines: WA25 (WiCell, WI) (Month 3-12).

Status: completed as of 10/31/2020.

Subtask 10: 3-D multicellular aggregates preparation (generation of auditory neuron spheroids).

Human embryonic cell lines: H1, H7, and H9 (WiCell, WI) (Month 3-12)

Status: completed as of 10/31/2020.

Major Task 3: *In vivo* development of supportive stem-cell niches.

Subtask 11: Optimize the number of PODS in nanofibrillar cellulose (NFC) hydrogels for 3-D multicellular aggregates (Month 2-4).

Status: in progress (75% completed as of 10/31/2020).

Subtask 12: 3-D multicellular aggregates preparation (generation of inner-ear organoids) Human embryonic cell lines: WA25 (WiCell, WI) (Month 13-36)

Status: in progress (50% completed as of 10/31/2020).

Subtask 13: 3-D multicellular aggregates preparation (generation of auditory neuron spheroids).

Human embryonic cell lines: H1, H7, and H9 (WiCell, WI).

Status: in progress (60% completed as of 10/31/2020).

Subtask 14: Transplantation of spheroids in the DTR⁺ mice inner ear using the volume-controlled system.

Status: completed as of 10/31/2020.

Subtask 15: Transplantation of organoids in the DTR⁺ mice inner ear using the pressure-controlled system

Status: in progress (30% completed as of 10/31/2020).

Subtask 16: Transplantation of spheroids in the DTR⁺ mice inner ear using volume-controlled system

Status: completed as of 10/31/2020.

Subtask 17: Transplantation of organoids in the DTR⁺ mice inner ear using pressure-controlled system

Status: in progress (30% completed as of 10/31/2020).

Subtask 18: Sacrifice and histological assessments of the chronically implanted DTR⁺ mice (1, 2, and 3 month survival periods)

Status: in progress (30% completed as of 10/31/2020).

Subtask 19: Sacrifice and histological assessments of the chronically implanted DTR⁺ mice (1-month survival period (n=40), 2-month survival period (n=40), and 3-month survival period (n=40)) (Month 4-33)

Status: in progress (40% completed as of 10/31/2020).

Subtask 20: Sacrifice and histological assessments of the DTR⁺ mice without DT injection (non-deafened control (n=40)) (Month 4-33).

Status: in progress (20% completed as of 10/31/2020).

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting.

1. Major Activities

Our Major Task 1 is to optimize delivery of hPSC-derived 3-D multicellular aggregates (3-D neuronal progenitor spheroids, neurons, and organoids) using an in vitro model. We have completed analysis of all experiments. The results have been published on our manuscript. Our Major Task 2 is to accomplish *in vitro* development of supportive stem-cell niches and major task 3 is in vivo development of supportive stem-cell niches. We have also compiled our result on the major task 2 and also on some part of the major task 3.

2. Specific Objectives

In Aim 1, we develop effective means of transplanting stem-cell derived SGNs into the scala tympani. In Aim 2, we will bioengineer and evaluate supportive niches to improve cell survival and neurite growth to improve the neural selectivity of CI electrodes. These aims are essential to the biohybrid CI, which leverages proven CI technology with the parallel transplantation of stem-cell-derived SGNs into the scala tympani.

3. Significant Result & Key outcomes

During this period, we published two peer-reviewed manuscripts on a major scientific journal—Tissue Engineering—Part A (3-year impact factor: 3.55) [1] and Acta Biomaterialia (5-year impact factor: 7.502) [2]. Therefore, we will substitute detailed descriptions of specific aspects of results and critical outcomes with these two articles. We will reference them under SOW in the text below. We have also presented our paper at the COMSOL Conference in 2019. We will also reference this proceeding in the text. Please refer Appendix for these copies of publications and proceedings.

Specific Aim 1:

Both Northwestern University IACUC and ACURO approvals have been recently updated. We are currently in good standing as of 10/31/2020 (Subtask 1) [2]. Three-dimensional (3-D) multicellular aggregates (inner ear organoids and otic neuronal progenitors (ONPs) spheroids) have been generated from human embryonic stem cell (hESC) lines (WA25, H1, H7, and H9) (Subtask 2 and 3) [1]. See

Fig .1, 2, Supplementary Fig. S3, and S4 for macroscopic images of hESC-derived ONP spheroids [1].

Our comparisons between a volume flow system and a pressure regulation system for transferring hESC-derived ONP spheroids and organoids demonstrated that a pressure regulation system resulted in fewer cell death, milder deformities (i.e., better survival from mechanical transfers) (Subtask 4 and 6) [1]. Therefore, we focused on evaluating the transfer of hESC-derived ONP spheroids/organoids using the pressure regulation system (subtask 5 and 7) [1]. Our significant findings indicate that hESC-derived ONP spheroids maintained the molecular and functional characteristics similar to those of ONP cells, which are upstream in the SGN lineage (Fig. 4 and 5) [1]. Moreover, our phenotypical, electrophysiological, and mechanical data suggest an optimal spheroid transplantation point after seven days of in vitro three-dimensional (3D) culture. (Fig. 6, 7, and 8) [1].

We have also developed a feasible transplantation protocol for these spheroids using a micropipette aided by a digital microinjection system. Our work demonstrates that ONP cells' transplantation in 3-D aggregates form into the inner ear through micropipette seven days after seeding for 3D spheroid culture is an expedient and viable method for stem cell-replacement therapies in the inner ear [1].

Specific Aim 2:

Multicellular aggregates (spheroids and organoids) have been generated (subtask 9 and 10) for this aim. Our data in Specific Aim 1 demonstrate a substantial effect of a sustained-release BDNF system and NFC hydrogel on hPSC-derived ON spheroids. In this aim, we focus on in vitro and in vivo development of stem cell niches.

For in vitro stem cell niche development (subtask 9 and 10), our results demonstrated that the transplanted hESC- derived ONP spheroids survived and neuronally differentiated into otic neuronal lineages in vitro (Fig. 1-7) [2]. For in vivo stem cell niche development (subtask 11-20), our results demonstrated that the transplanted hESC- derived ONP spheroids survived and neuronally differentiated into otic neuronal lineages in vivo and also extended neurites toward the bony wall of the cochlea 90 days after the transplantation without the use of immunosuppressant medication (Fig 8, 9) [2].

In summary, our data in vitro and in vivo presented here provide sufficient evidence that we have established a robust, reproducible protocol for in vivo transplantation of hESC-derived ONPs to the inner ear. Using our protocol to create an artificial stem cell niche in the inner ear, it is now possible to integrate transplanted hESC-derived ONPs further and work toward achieving functional auditory neurons generated from hESCs. Our findings suggest that the provision of an artificial stem cell niche can be a future approach to stem cell-replacement therapy for inner-ear regeneration.

Reference for this section

- [1] Heuer RA, Nella KT, Chang HT, Coots KS, Oleksijew AM, Roque CB, Silva LHA, McGuire TL, Homma K, Matsuoka AJ. Three-Dimensional Otic Neuronal Progenitor Spheroids Derived from Human Embryonic Stem Cells. *Tissue Engineering –Part A*. 2020 Aug 7. doi: 10.1089/ten.TEA.2020.0078. Epub ahead of print. PMID: 32580647.
- [2] Chang HT, Heuer RA, Oleksijew AM, Coots KS, Roque CB, Nella KT, McGuire TL, Matsuoka AJ. An engineered three-dimensional stem cell niche in the inner ear by applying a

nanofibrillar cellulose hydrogel with a sustained-release neurotrophic factor delivery system. *Acta Biomaterialia*. 2020 May;108:111-127. doi: 10.1016/j.actbio.2020.03.007. Epub 2020 Mar 7. PMID: 32156626; PMCID: PMC7198367.

- [3] Bharadwaj S, Oleksijew A, Fernandez J, Chang H-T, Hauer R, Richter C-P, et al. COMSOL Conference 2018 Proceeding. A 3D simulation of the diffusion profile of brain-derived neurotrophic factor in the inner ear using the COMSOL Multiphysics® software. COMSOL; 2018. Available: <https://www.comsol.com/paper/a-3d-simulation-of-the-diffusion-profile-of-brain-derived-neurotrophic-factor-in-63862>
- [4]. Bharadwaj S, Oleksijew A, Fernandez J, Chang H-T, Hauer R, Richter C-P, et al. COMSOL Conference 2019 Proceeding. COMSOL Multiphysics® Finite element model of the diffusion profile of brain-derived neurotrophic factor: biological validation through a microfluidic device. COMSOL; 2019. Available: <https://uk.comsol.com/paper/a-comsol-multiphysics-finite-element-model-of-the-diffusion-profile-of-brain-der-81011>

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Walk 4 Hearing (Hearing Loss Association on America), Chicago, Oct 4, 2020 (Canceled due to the COVID-19 pandemic).

I would like to continue involvement in Walk 4 Hearing in Chicago area. I am also planning to give a lecture at American Tinnitus association (Chicago branch) regarding emerging treatment of sensorineural hearing loss.

What you plan to do during the next reporting period to accomplish the goals and objectives?

We will focus on in vivo part of our project (i.e., Specific Aim 2). More specifically, we are planning to perform transplantation of hPSC-derived 3-D multicellular aggregates. We will also complete subtask 11 in that we need to optimize the number of PODS in NFC hydrogels. We will also need to increase the number of transplanted mice to complete subtask 11-20. While we do not know how the COVID-19 pandemic impact our research, we feel that it is realistic to complete these subtask in a reasonable time frame as outlined in our updated Quadchart (See Appendix).

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.” Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Both the COVID-19 pandemic and recent civil unrest in the downtown Chicago area have significantly impacted this study's progress.

1) Due to social distancing policy implemented by Northwestern university, we are only allowed to have fewer than four people in our lab space. We are currently using two shifts (7 am-3 pm and 3 pm-10 pm). The newly implemented shift limits our use of lab space as well as our core facilities in the Chicago campus.

2) Due to the COVID-19 pandemic and recent civil unrest in Chicago, it is no longer deemed to be safe to come to work by public transportation. Therefore, our lab members require to come to work by car, which incurs additional cost for parking. This has impacted the progress of the study as the lab members are not eager to come to work by car and they are naturally trying to work from home.

3) In terms of administration, the university is still being operated remotely, and therefore, the use of core facilities is limited. The reservation is always full, and the access is restricted.

4) Animal facilities are also running with limited capacity. They are gradually back to full capacity as of 10/31/2020.

5) Both psychological/emotional distress by the COVID-19 pandemic and future uncertainty including job security have resulted in requiring psychological counseling for some of our lab members. They need to take sick leave more often to maintain their mental health.

Unfortunately, we do not have any definite plans to address these problems at this point (10/31/2020).

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Authors: Chang HT, Heuer RA, Oleksijew AM, Coots KS, Roque CB, Nella KT, McGuire TL, Matsuoka AJ.

Title: *An Engineered three-dimensional Stem Cell Niche in the Inner Ear with Nanofibrillar Cellulose Hydrogel and a Polyhedrin Delivery System*

Journal: Acta Biomaterialia

Volume: 108

Year: 2020

Page numbers: 111-127, doi: 10.1016/j.actbio.2020.03.007

Status of publication: Published

Acknowledgement of federal support: yes

Authors: Heuer RA, Nella KT, Chang HT, Coots KS, Oleksijew AM, Roque CB, Silva LHA, McGuire TL, Homma K, Matsuoka AJ.

Title: *Three-Dimensional Otic Neuronal Progenitor Spheroids Derived from Human Embryonic Stem Cells*

Journal: Tissue Engineering Part A

Year: 2020

Page numbers: doi: 10.1089/ten.TEA.2020.0078.

Status of publication: Published (Epub ahead of print)

Acknowledgement of federal support: yes

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic*

information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

CONFERENCE PAPERS (International)

ISSCR (International Society of Stem Cell Research) 2020, Boston, MA, USA. June 24th-28th, 2020 (Virtual meeting)

TSC317: Matsuoka AJ., Nella KT, Bharadwaj S, Heuer RA, Chang HT, Coots KS, Oleksijew AM, Roque CB, Silva LHA, McGuire TL, Peter S., Richter C-P. Three-Dimensional Finite Element Model for the Diffusion Kinetics of Neurotrophic Factors in the Inner Ear: Biological and Chemical Validation.

TSC284: Heuer RA, Chang HT, Matsuoka AJ., Nella KT, Coots KS, Oleksijew AM, Roque CB, Silva LHA, McGuire TL, Homma K, Matsuoka AJ. Otic Neuronal Differentiation of Three-Dimensional Spheroids derived from Human Pluripotent Stem Cells: Characterization toward In vivo Transplantation*

TSC285: Chang HT, Heuer RA, Matsuoka AJ., Nella KT, Coots KS, Oleksijew AM, Roque CB, Silva LHA, McGuire TL, Homma K, Matsuoka AJ. Engineering a Three-Dimensional Stem Cell Niche in the Inner Ear by Applying a Nanofibrillar Cellulose Hydrogel with a sustained-release Neurotrophic Factor Delivery System*

CONFERENCE PAPERS (National)

COMSOL 2019, Boston, MA, USA. Oct 2th-4th, 2019.

Bharadwaj S, Oleksijew A, Fernandez J, Chang H-T, Hauer R, Richter C-P, Matsuoka AJ. COMSOL Conference 2019 Proceeding. COMSOL Multiphysics® Finite element model of the diffusion profile of brain-derived neurotrophic factor: biological validation through a microfluidic device. COMSOL; 2019. Available: <https://uk.comsol.com/paper/a-comsol-multiphysics-finite-element-model-of-the-diffusion-profile-of-brain-der-81011>.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

U.S. Patent Application (filed)

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: Akihiro Joseph Matsuoka, M.D., D.M.Sc., Ph.D. FACS
Project Role: Principal Investigator
Researcher Identifier: <https://orcid.org/0000-0002-1066-6514>
Nearest person month worked: 3.0
Contribution to Project: Dr. Matsuoka has performed the overall planning of the project, defining experimental directions and designs, interpreting data, communicating with his co-investigator, statistical analysis, and reporting project results to the sponsor and the scientific community in the form of progress reports, papers, and presentations.

Name: Kevin Nella, B.S.
Project Role: Research Technician
Researcher Identifier: N/A
Nearest person month worked: 12
Contribution to Project: This individual performed nanofibrillar cellulose gel generation, incorporation of POS crystals into Growdex.

Name: Christian Roque, B.S.
Project Role: Research Technician
Researcher Identifier: N/A
Nearest person month worked: 12
Contribution to Project: This technician performed a Live-Dead cell assay, nanofibrillar cellulose gel generation, incorporation of POS crystals into Growdex, and immunocytochemistry.

Name: Luisa Helena Andrade da Silva
Project Role: Postdoctoral fellow
Researcher Identifier: N/A
Nearest person month worked: 12
Contribution to Project: This individual performed mouse surgery and Immunohistochemistry.

Name: Shreyas Bharadwaj
Project Role: Medical student (research rotation)
Research Identifier: N/A
Nearest person month worked: 2
Contribution to Project: This individual performed designing computational model for Aim 2

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

See page 55 (Appendix).

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Three-Dimensional Otic Neuronal Progenitor Spheroids Derived from Human Embryonic Stem Cells

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Stem cell-replacement therapies have been proposed as a potential tool to treat sensorineural hearing loss by aiding the regeneration of spiral ganglion neurons (SGNs) in the inner ear. However, transplantation procedures have yet to be explored thoroughly to ensure proper cell differentiation and optimal transplant procedures. We hypothesized that the aggregation of human embryonic stem cell (hESC)-derived otic neuronal progenitor (ONP) cells into a multicellular form would improve their function and their survival *in vivo* post-transplantation. We generated hESC-derived ONP spheroids—an aggregate form conducive to differentiation, transplantation, and prolonged cell survival—to optimize conditions for their transplantation. Our findings indicate that these cell spheroids maintain the molecular and functional characteristics similar to those of ONP cells, which are upstream in the SGN lineage. Moreover, our phenotypical, electrophysiological, and mechanical data suggest an optimal spheroid transplantation point after 7 days of *in vitro* three-dimensional (3D) culture. We have also developed a feasible transplantation protocol for these spheroids using a micropipette aided by a digital microinjection system. In summary, the present work demonstrates that the transplantation of ONP cells in spheroid form into the inner ear through micropipette 7 days after seeding for 3D spheroid culture is an expedient and viable method for stem cell replacement therapies in the inner ear.

Keywords: human embryonic stem cells, stem cell niche, spiral ganglion neurons, inner ear, stem cell replacement therapy, spheroid, three-dimensional culture

Impact Statement

Sensorineural hearing loss affects millions of people worldwide. Although inner ear stem cell replacement therapies offer a promising method to mitigate this hearing loss by aiding in the regeneration of spiral ganglion neurons, few *in vivo* studies have been performed in this area. By providing detailed cell spheroid characterization, determining an optimal development stage for transplantation, and establishing a reliable and reproducible transplantation protocol, we aimed to provide the necessary details to bridge the gap between *in vitro* experiments and *in vivo* studies of stem cell replacement therapies in the inner ear.

Introduction

STEM CELL REPLACEMENT THERAPIES in the inner ear may aid regeneration of spiral ganglion neurons (SGNs) and mitigate the effects of sensorineural hearing loss (SNHL), which typically results from destruction of hair cells followed by loss of SGNs.^{1–3} Recently, human embryonic stem cells (hESCs) have become invaluable tools to study tissue

regeneration because they can differentiate toward an otic neuronal lineage, the precursor to SGNs.^{4–7} Despite promising initial results, however, low engraftment and immature otic neuronal differentiation still pose significant challenges. These challenges are at least partially due to the inner ear's inhospitable environment, which consists mainly of bone and the nutrient-poor perilymph.^{8–10} As a result, transplanted hESC-derived cells cannot interact with native surrounding

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cells to regulate and promote proliferation, differentiation, and survival. Recent tissue engineering advancements have enabled the generation of more hospitable artificial micro-environments (i.e., stem cell niches) with the necessary cellular components, extracellular matrices (ECMs), and secreted factors to support transplanted cells.^{11–13}

The use of three-dimensional (3D) cell culture techniques to replicate these niches have gained traction: for example, the formation of cellular aggregates (e.g., spheroids) enhances cellular functional properties.^{14,15} It has been reported that the formation of spheroids can up-regulate the secretion of neurotrophic factors.¹⁶ Furthermore, the formation of hESC spheroids demonstrably enhances the secretion of ECM components, including fibronectin and laminin, which can promote hESCs' neuronal differentiation, prolong cell survival, and provide better protection from neurotrophic factor deprivation.^{17–19} In addition, neural cells in particular have been grown successfully in 3D culture, which provides such benefits over flat culture as increased neurite outgrowth.²⁰ Lastly, it is known that neural cells can self-assemble into spheroid structures without the requirement for a provided matrix.^{21,22}

However, the precise impact of 3D spheroidal cellular organization and structural environment on hESC-derived otic neuronal progenitors (ONPs) has not been investigated. We hypothesized that the formation of hESC-derived ONP spheroids facilitates otic neuronal differentiation and improves cell viability after transplantation in the inner ear, as an alternative to single-cell transplantation. Since *in vivo* animal studies are expensive, low-throughput, and unpredictable, 3D spheroids must be extensively analyzed before transplantation to ensure maintenance of proper cell characteristics and to determine the ideal time for transplantation. In

particular, hESC-derived ONP spheroids must be examined to ensure that they maintain the ONP characteristics necessary for regeneration of SGNs. Moreover, a protocol must be developed to establish that these spheroids can be effectively transplanted at the optimum point for maximum cell survival.

Therefore, we first constructed spheroids from pluripotent hESCs. These spheroids were characterized to evaluate cellular, molecular, mechanical, and functional properties to ensure that the cells displayed neuronal progenitor characteristics and to determine the best transplantation point. We also investigated specific parameters for a spheroid transfer procedure to efficiently transplant spheroids into the inner ear with minimum stress on the cells. Results indicate that hESC-derived ONP spheroids possess attractive characteristics for stem cell replacement therapies in the inner ear and that culturing spheroids for 7 days *in vitro* allows sufficient development of ONPs before transplantation.

Materials and Methods

Generation of hESC-derived ONP spheroids

hESC lines H1, H7, and H9 (WiCell, Madison, WI) were differentiated toward an otic neuronal lineage according to our previously described protocol (Fig. 1).⁵ Upon reaching the late ONP stage (Fig. 1) on day 25 (D25), cells were passaged to a 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (U-bottom, #7007; Corning Life Science, Tewksbury, MA) and seeded at either 25,000; 50,000; or 75,000 cells per well. The plate was then centrifuged at 201 relative centrifugal force for 10 min and left incubating at 37°C for 7–28 days. For our mechanical assessment, additional late-stage ONP spheroids were generated in a different culture system by seeding 1.8–2.5 million cells in an

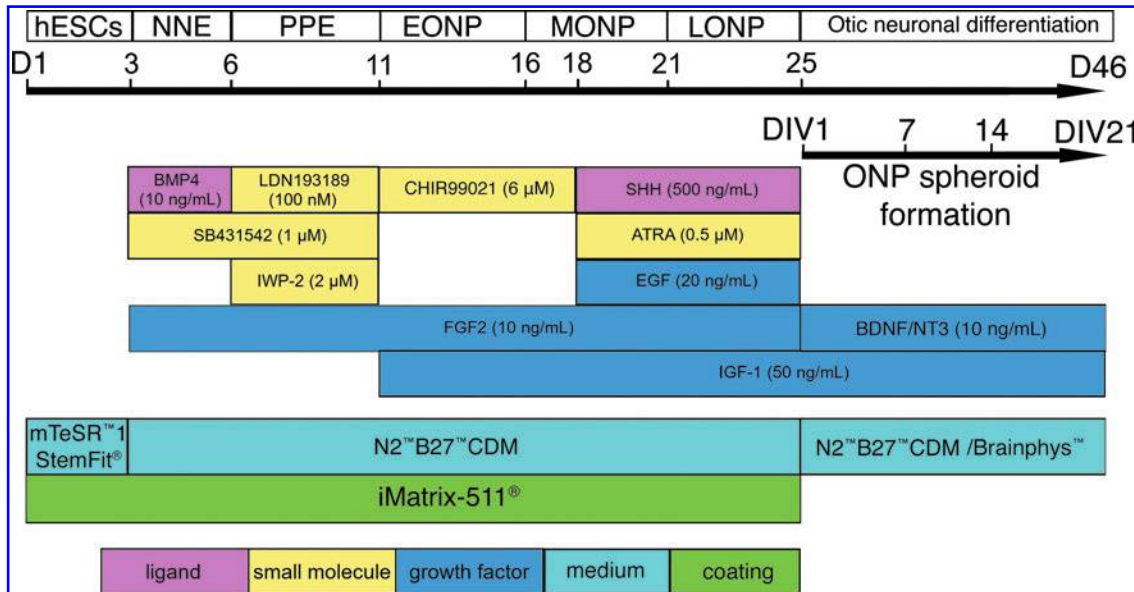


FIG. 1. Schematic summary of the protocol and timeline for generating late-stage ONPs from undifferentiated hESCs. Developmental stage is shown at the *top*, with key treatments shown below. D, day of cell culture; DIV, day *in vitro* after three-dimensional seeding; hESCs, human embryonic stem cells; NNE, non-neuronal 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. Color images are available online.

EZSPHERE™ culture plate (Cat#:4000-900SP, Nacalai USA, San Diego, CA, manufactured by AGC Techno Glass, Shizuoka, Japan), which has 2300 microwells designed for spheroid formation on its surface. Spheroid day *in vitro* 1 (DIV 1) was defined as the first day when late-stage ONPs were transferred into a spheroid-forming plate. Spheroids generated by U-bottom plate were imaged regularly after formation with a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Each week, spheroids seeded at 50,000 cells per well were fixed for 20–30 min with 4% paraformaldehyde (Cat#: 158127; Millipore Sigma, Burlington, MA) and rinsed twice with Dulbecco's phosphate-buffered saline (DPBS) (Corning, Life Sciences). Fixed spheroids were stored in DPBS at 4°C before use. Spheroids seeded at 50,000 cells per well were used for all experiments, except for the analyses of diameter, circularity, and roundness.

Image acquisition and cell quantification

Samples were imaged with a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany). For quantitative analyses on hESC-derived ONP spheroids, the average diameter of each spheroid was measured. Here, the geometric mean diameter of a spheroid (D_g) is defined as follows:

$$D_g(m) = \sqrt[3]{a * b}$$

where a and b are the major and minor axes of the diameter, respectively. Using this equation, we measured the spheroids' diameters using Microscope Imaging Software (Leica Microsystems, Inc., Buffalo Grove, IL).

Sphericity and roundness are metrics to measure the compactness of a shape.²³ Sphericity describes the similarity to pure spheroidal geometry for each spheroid. Given that we measured the diameter of a spheroid in single two-dimensional (2D) images, we used the circularity index (C), which is the 2D equivalent of the true 3D spheroid index and ranges from 1 (perfect sphere or circle) to 0 (elongated shape).²⁴ For the circularity index,

$$C = \frac{4\pi S}{P^2}$$

where S is the spheroid surface area as measured on a 2D spheroid image with the largest diameter, selected from the Z-stack images of each spheroid. P is the spheroid perimeter. We also used a roundness measurement to describe the similarity of the spheroid images to a perfect circle (the shape's gross feature). Roundness was defined as follows²⁴:

$$R = \frac{4S}{\pi a^2}$$

where S is the surface area and a is the major axis of the diameter.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling

DNA fragmentation was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining to evaluate cell viability in spheroids. At weekly time points, spheroids seeded at 50,000 cells per well were

fixed, frozen, and sliced into 10 μ m sections on a Leica CM3050 S cryostat (Leica). Center sections were treated with the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to manufacturer's instructions to detect the DNA fragmentation indicative of cell death. Samples were then imaged with a Leica TCS SP5 confocal microscope. Cells with TUNEL staining restricted to the nuclei as confirmed by DAPI double staining were considered apoptotic (TUNEL-positive); diffusely TUNEL-stained cells with no apoptotic morphology were excluded based on previously used criteria.²⁵ TUNEL-positive cells were counted and expressed as a ratio of total cells in sections counterstained with DAPI for each region. Data are expressed as percent DAPI-stained cells.

Three-dimensional immunocytochemistry

Fixed spheroids were blocked overnight at room temperature in 10% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) with 0.1% Triton X-100 (Sigma-Aldrich) in DPBS before incubation with primary antibodies (Supplementary Table S1). After 2 days, spheroids were washed three times with DPBS with 0.1% Triton X-100 and then incubated in secondary antibody solution (Supplementary Table S2) for 3 days. Spheroids were counterstained with TOTO-3 iodide (1:5000–1:10,000 in DPBS, T3604; Thermo Fisher Scientific, Waltham, MA) for 45 min followed by a final wash. Immunocytochemistry controls were performed as previously described.^{26,27} Primary antibody controls were obtained with hESC-derived ONPs with a second primary antibody to the same antigen.²⁶ Secondary antibody controls were also obtained each time multiple primary antibodies were used.²⁶ Labeling controls (detection controls) were performed for a sample from each batch of hESC culture (Supplementary Fig. S1).²⁶ Samples were imaged with a Leica TCS SP5 confocal microscope (Leica). The quantification of immune-positive cells has been previously described in detail (Supplementary Data).^{5,6}

Neurite arborization assay

The pan-neuronal marker β -III tubulin was used to calculate neurite arborization area. Individual confocal optical Z-stack sections were examined through the extent of each spheroid at the observation time point using ImageJ ver. 2.0.0-rc-69/1.52p (National Institute of Health, Bethesda, MD).²⁸ The watershed algorithm was extensively used to identify individual nuclei.²⁹ Each Z-stack photomicrograph was transformed into an 8-bit grayscale.^{30,31} Quantification was assessed after defining a threshold for background correction using the "MaxEntropy" method of the AUTO THRESHOLD plugin.^{32,33} Arbor surface area (μ m²) was calculated using an established protocol and was normalized with each Z-stack spheroid surface area, as spheroid sizes varied depending on the culture length.³²

Whole-cell patch-clamp recordings

To test the efficacy of certain culture conditions for neuronal differentiation, ONP spheroids were cultured in early neuronal stage differentiation medium as previously described.⁵ ONP spheroids were cultured with a polyhedrin delivery system (PODS®, #PPH1-50; Cell Guidance Systems, Cambridge,

United Kingdom) carrying brain-derived neurotrophic factor (BDNF), designed to release BDNF at a regular rate into medium. Spheroids were also cultured with GrowDex-T® (#200103010, UPM-Kymmene Corporation, Helsinki, Finland), a nanofibrillar cellulose hydrogel used to simulate an ECM. PODS were supplied at 800,000 per spheroid. At given time points, these spheroids were examined by patch clamp. Standard voltage- and current-clamp recordings were performed using an Axopatch™ 200B amplifier (Axon Instruments, Inc., Union City, CA) following established protocol (Supplementary Data).³⁴

Mechanical characterization of ONP spheroids

To examine the mechanical properties of hESC-derived ONP spheroids, spheroids formed using both a 96-well Clear Round Bottom Ultra-Low Attachment Microplate (U-bottom) and an EZSPHERE culture plate were grown until DIV 4, at which point spheroids were immobilized and tested with a single indentation protocol using a Piuma nanoindenter (Optics11, Amsterdam, Netherlands). Subsequent nanoindenter testing was performed on DIV 8 and DIV 11. Control data were obtained using mouse brain samples (Supplementary Data). MATLAB (version 9.5.0 1067069 (R2018b) Update 4, The MathWorks, Inc., Natick, MA) was used to analyze the load–displacement data. The Hertz method was used for analysis because the use of the loading portion of the load–displacement curve is far more suitable for soft biological samples.³⁵ The Hertz model assumes a linear elastic material response. The elastic Young’s modulus was calculated as follows:

$$E = \frac{3(1 - \nu^2)P}{4R^{1/2}h^{3/2}}$$

where E is the elastic Young’s modulus, h is the cantilever deflection (nm), R is the radius of the spherical indenter tip (μm), and ν is Poisson’s ratio (Supplementary Data).

ONP spheroid mock transplantation using a micropipette

For mock transplantation of hESC-derived ONP spheroids, we used a cell-holding system originally developed for *in vitro* fertilization, designed to capture cell aggregates at the tip of a micropipette with negative pressure. Spheroid transfer pipettes were pulled from borosilicate glass with a Sutter P-97 Flaming/Brown Micropipette Puller (Sutter Instrument, Novato, CA). Micropipettes were then broken to size using the score and break technique, with a Sutter Instruments Ceramic Tile for Scoring Glass (NC9569052; Sutter Instrument) used to score the glass and allow for a clean break. Pipettes were not fire polished. Please also see the Supplementary Data for details.

The transplantation procedure was simulated using a Xenonworks Digital Injector (Sutter Instrument) system with micropipettes in a range of sizes. For transfer, the pipette tip was placed at the surface of a spheroid and the negative pressure manipulated to hold the spheroid in place. Upon moving the pipette tip with the captured spheroid out of the initial culture medium to the desired area of transfer, positive pressure released the spheroid from the micropipette. Spheroids were

placed in a 96-well culture dish filled with culture medium to simulate placement into the fluid-filled scala tympani. A range of micropipette sizes was used to determine the optimal conditions for transferring spheroids with a low amount of stress. Pressures ranged from -124 to $+55$ hPa.

Live/dead assay of transferred spheroids

After transfer, a subpopulation of spheroids was cultured in medium with the Invitrogen LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA) for 2–3 h according to manufacturer’s protocol. Positive and negative controls are shown in Supplementary Figure S2. Images of the surface of each transferred spheroid were taken using a Leica TCS SP5 confocal microscope. Using ImageJ, a threshold was set on each image to distinguish live and dead cells. Images were made binary and the total area of cells was measured for each category, live and dead. Cell survival was quantified as the ratio of live cell area to combined live and dead cell area.

Nonlinear curve-fitting analysis

Data were transferred to JupyterLab (version 1.0.2) (Project Jupyter, Worldwide) as a comma-separated value file for a nonlinear curve-fitting analysis using Python (version 3.8.0) (Python Software Foundation, Wilmington, DE). The following Python libraries were used for the analysis: Scipy, Numpy, Matplotlib, and Seaborn.^{36–39} A monoexponential equation was used to fit data using the “scipy.optimize.curve_fit” and “scipy.optimize.least_squares” modules in Scipy. These two modules use the Levenberg–Marquardt algorithm based on the Gauss–Newton method.^{40,41} The algorithm returns a vector of estimated coefficients by the nonlinear curve-fit using the model specified by the following equation:

$$y(t) = (y_0 - y_f)e^{-t/\tau} + y_f$$

where y is an estimated spheroid diameter, t is time, τ is the decay constant, y_0 is the initial diameter at $t=0$, and y_f is the spheroid diameter at equilibrium. The coefficient of determination (R^2) and the residual standard error (RSE) were calculated for evaluating goodness of fit. On each analysis, the decay constant and the half-life time were calculated. Given that the initial time point of the curve fitting was DIV 1 instead of DIV 0, we also defined adjusted half-life time ($t_{1/2}(\text{adj})$) as follows:

$$t_{1/2}(\text{adj}) = t_{1/2} + 1$$

Statistical analyses

When appropriate, and as indicated in each figure, statistical analysis was performed. The value of “ n ” represents biological replicates. For instance, in our measurement of elastic Young’s modulus, it was necessary to confirm that each measurement was reliably performed and as such, we used three to five technical replicates for the measurement on each spheroid to determine one biological replicate (i.e., n).^{42,43} The sample size (n) is stated in each figure caption. Experimental values are typically expressed as mean $\pm$ standard error

(SE). Median, standard deviation (SD), and first and third quartiles were used in some cases. All statistical analyses were performed with R (version 4.0.0. 2020-04-24, Vienna, Austria).⁴⁴ Normal distributions were assumed unless mentioned otherwise. Analysis of variance (ANOVA) was performed with a Tukey's Honest Significant Different (HSD) *post hoc* test. Shapiro–Wilk normalization tests and F-tests were also performed. *p* values smaller than 0.05 were considered statistically significant.

Results

Characterization of hESC-derived ONP spheroids

Human ESC-derived ONPs were seeded at three different densities—25,000; 50,000; and 75,000 cells per well—and allowed to grow for 3 weeks. All spheroids consistently decreased in size (Fig. 2A), and the rate of shrinkage slowed over time. Images of prespheroid cultures taken on spheroid day *in vitro* (DIV) 1 an hour after seeding show a primarily monolayer culture, with a 3D structure beginning to form around the culture perimeter of each well (white arrow). Figure 2B shows the diameter of hESC-derived ONP spheroids at six different time points. Nonlinear mono-exponential curve fit was performed on each cell density (Fig. 2C). On each analysis, the decay constant (τ) and the half-life time ($t_{1/2}$) of a fitted curve were calculated. The R^2 and RSE values were also calculated for evaluating goodness of fit (Table 1). The decay constant was greater at the seeding cell density of 75,000 cells/well than 25,000 cells/well, suggesting that greater cell density leads to a faster decay. Half-life times ($t_{1/2}$) of fitted curves for all cell densities showed an average of 2.07 ± 0.374 days (mean $\pm$ SD), whereas $t_{1/2}$ (adj) had an average of 3.07 ± 0.374 days. Finally, the R^2 and RSE values, along with the visual examination of fitted curves, demonstrated reasonable curve fit of the three cell densities. Figure 2D and E show circularity and roundness, respectively, of spheroids at six different time points. The mean circularity index of spheroids at the cell density of 25,000 cells/well was significantly lower than the other two conditions ($p < 0.05$) (Fig. 2D). Circularity indices and roundness slowly declined after DIV 7.

TUNEL assay

TUNEL-positive cells displaying apoptotic morphology were present in spheroids at all time points (Fig. 3A). However, quantitative analysis (Fig. 3B) indicated that the ratio of such cells increases significantly after DIV 14, with nonconcentric configuration.

Immunocytochemistry

Immunocytochemistry was performed on spheroids to assess ONP characteristics (Fig. 4A). All spheroids highly expressed GATA3, an otic lineage marker; PAX8, another otic lineage marker that is positive for the otic placode and vesicle; and pan-neuronal marker β -III tubulin at DIVs 3, 7, 14, and 21.^{45–50} Spheroids also expressed the neuronal progenitor marker nestin at DIVs 3 and 7, but nestin expression was significantly reduced after DIV 14 (Fig. 4B).⁴⁵ Glutamatergic receptor 2 (GLUR2), a neuronal marker found in SGNs, and vesicular glutamate transporter 2 (VGLUT2), a marker specific to glutamatergic neurons, showed significantly low expression at DIV 3, but

high expression after DIV 7.^{51,52} Finally, a neurite arborization assay showed significantly less axonal branching at DIV 3 compared with DIVs 7, 14, and 21 (Fig. 4C).

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were performed to investigate the spheroids' electrophysiological properties. Average resting membrane potentials in DIV 5 and 7 ONP spheroids (mean $\pm$ SD) were -19.0 ± 7.9 mV ($n = 7$) and -28.5 ± 7.8 mV ($n = 8$), respectively. While there were virtually no current activities in whole-cell voltage-clamp recordings on DIV 5 (Fig. 5Aa), DIV 7 spheroids showed voltage-activated current efflux above -20 mV (Fig. 5Bb), indicative of a large voltage-gated potassium (Kv) channel activity. Consistently, whole-cell current-clamp recording in DIV 7 spheroids showed clearly detectable transmembrane potential (V_m) repolarization after current-induced cell depolarization (Fig. 5Bc), an essential electrophysiological characteristic found in all types of neurons. In contrast, DIV 5 spheroids indicated no signs of V_m repolarization (Fig. 5Ac).

Mechanical properties of hESC-derived ONP spheroids

Figure 6A shows a representative force curve obtained from an hESC-derived ONP spheroid using the Piuma nanoindenter. Note the smoothing effect of the Hertzian curve fit to the raw data. Note also that the indentation depth used in this study was kept below $10 \mu\text{m}$ to avoid detecting the substrate on which the spheroids were resting.^{21,35} As shown in Figure 6B, elastic Young's modulus increased over time, but did not increase at equal rates for the two culture plates. The Young's modulus of spheroids from both plates at DIV 4 is not significantly different from that of the mouse brain control tissue. By DIV 8, the spheroids formed in U-bottom plates had a Young's modulus significantly greater compared with the mouse brain tissue and were stiff enough to hold up to the stress of mock transplant. In contrast, the spheroids formed in an EZSPHERE plate took until DIV 11 to reach this point; at DIV 8, the elastic Young's modulus of U-bottom spheroids is significantly greater compared with the EZSPHERE spheroids.

Simulated transplantation of ONP spheroids using a pressure-based delivery system

Our micropipette transfer system is depicted in Figure 7, which shows spheroids held on or within a micropipette by means of negative pressure ($\Delta P = 5$ hPa). Using this system, mock transplantation was performed with a number of spheroids and a range of micropipette diameters. Micropipettes were chosen with inner diameters (ID) covering a range from 10% to 85% of the diameters of the spheroids (based on values used for *in vitro* fertilization).^{53,54} (Supplementary Figs. S3 and S4 show the photomicrographical images used to determine this range.) Figure 8A shows the pickup and release pressures necessary to capture and transfer spheroids; the pressure changes required decrease as micropipette ID increases (from 175 to $385 \mu\text{m}$) (Supplementary Fig. S5 for a pilot study supporting this trend). The pressure data in Figure 8B indicate that the closer the size of the micropipette to the size of the spheroid, the less negative pressure required to capture the spheroid and the less

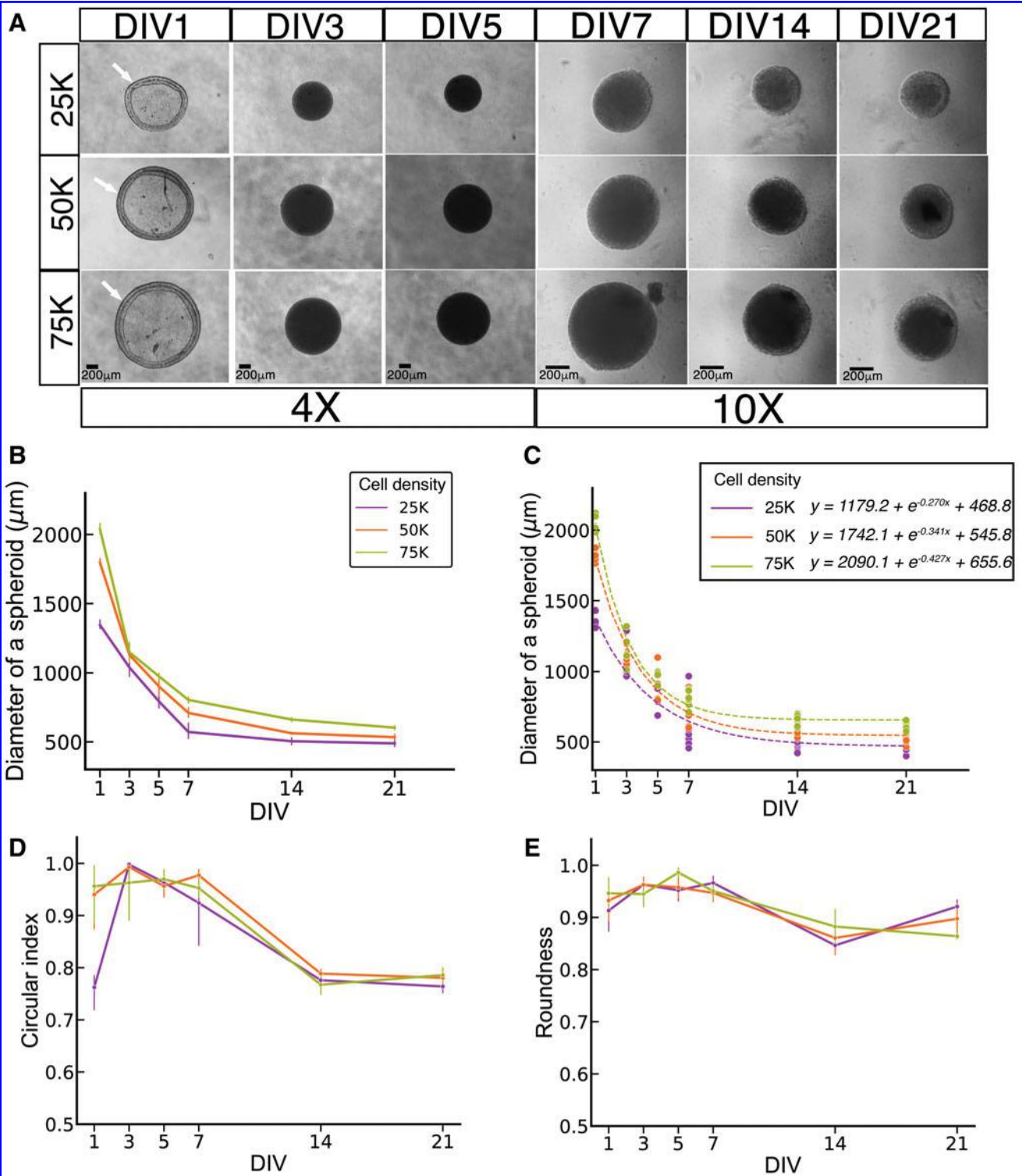


FIG. 2. Development of hESC-derived ONP spheroids over a 3-week culture period. **(A)** Phase-contrast microscopic photomicrographs of hESC-derived ONP spheroids at DIV 1, 3, 5, 7, 14, and 21 with original seeding densities of 25,000 (25K); 50,000 (50K); and 75,000 (75K) cells/spheroid. **(B)** The diameter of hESC-derived ONP spheroids at DIV 1, 3, 5, 7, 14, and 21 ($n=6$, DIV 1, 3, 5; $n=15$, DIV 7, 14, 21). Error bar: standard error. **(C)** Nonlinear curve fit of the diameter of hESC-derived ONP spheroids with seeding density of 25,000; 50,000; and 75,000 cells/spheroid. **(D)** Circularity of hESC-derived ONP spheroids at six different time points ($n=6$, DIV 1, 3, 5; $n=15$, DIV 7, 14, 21). Error bar: standard error. **(E)** Roundness of hESC-derived ONP spheroids at six different time points ($n=6$, DIV 1, 3, 5; $n=15$, DIV 7, 14, 21). Error bar: standard error. Color images are available online.

TABLE 1. THE DECAY CONSTANT (τ), HALF-LIFE TIME ($t_{1/2}$), ADJUSTED HALF-LIFE TIME ($t_{1/2}(\text{adj})$), CORRELATING COEFFICIENT (R^2), AND RESIDUAL STANDARD ERROR FOR THE NONLINEAR MONOEXPONENTIAL FITTING CURVE FOR THE DIAMETER CHANGE OF hESC-DERIVED ONP SPHEROIDS OVER TIME WITH THREE DIFFERENT CELL-SEEDING DENSITIES (25,000 (25K); 50,000 (50K); AND 75,000 (75K) CELLS/ WELL)

	Decay constant (τ)	Half-life time ($t_{1/2}$)	Adjusted half-life time ($t_{1/2}(\text{adj})$)	R^2	RSE
25K/well	0.27	2.56	3.56	0.985	444.6
50K/well	0.34	2.01	3.01	0.997	613.9
75K/well	0.42	1.65	2.65	0.987	683.7
M $\pm$ SD	0.34	2.07 $\pm$ 0.374	3.07 $\pm$ 0.374	0.987 $\pm$ 0.005	581.4 $\pm$ 99.5

M, mean; SD, standard deviation.

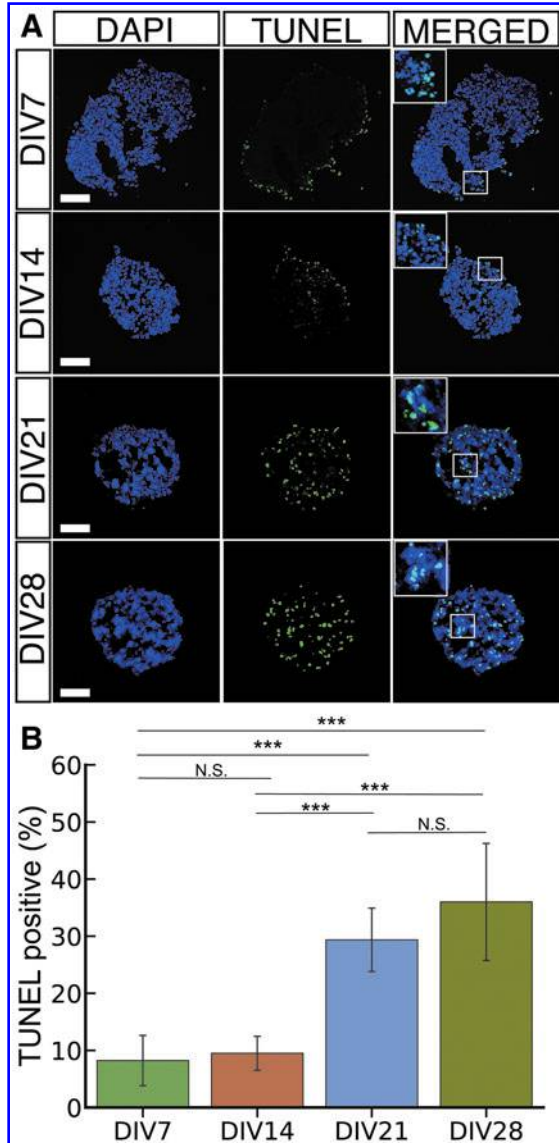


FIG. 3. Terminal deoxynucleotidyl transferase dUTP nick-end labeling. (A) Representative fluorescent images of DAPI and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of hESC-derived ONP spheroids at DIV 7, 14, 21, and 28. (B) Quantitative analysis of TUNEL-positive cells at DIV 7, 14, 21, and 28 ($n=6$ per each group), expressed as percent DAPI-stained cells. Error bar: standard error. ***Indicates statistical significance with one-way ANOVA with Tukey's HSD *post hoc* test ($p<0.001$). N.S., not significant. Color images are available online.

positive pressure required for release. A nonlinear mono-exponential curve fit was performed on the pressure data (Fig. 8B, green line). The decay constant (τ) was 2.43 and the half-life time ($t_{1/2}$) was 0.286. R^2 and RSE were 0.9876 and 16.31, respectively, indicating good fit.

With this trend in mind, cell survival was analyzed for spheroids in each of three “zones”: Zone I spheroids were transferred with micropipette of IDs 10–30% of the spheroid diameters, Zone II with micropipettes of ID 30–50% of spheroid diameters, and Zone III with micropipettes of ID 50–85% of spheroid diameters. All spheroids showed some proportion of dead cells (Fig. 8C), but for Zone I and II spheroids, dead cell populations were clustered particularly around the point of contact of the pipette (Fig. 8C, white dotted lines: the third column in Zone I and II). Cell survival was assessed as the ratio of live cell area compared with total cell area (Fig. 8D). In Figure 8D, the SD of live cell area in Zone I is greater compared with Zone II and III. To verify this difference statistically, a Shapiro–Wilk normality test was performed first to confirm the normal distribution of these data ($p=0.987$ [Zone I], 0.845 [Zone II], and 0.876 [Zone III], respectively). After confirming the normal distribution, we performed F-tests between Zone I and Zone II, and Zone I and Zone III. The p -value of the F-test indicated $p<0.05$ (0.015 for Zone I and II, 0.012 for Zone I and III), demonstrating that the SD in Zone I was statistically larger compared with Zone II or Zone III. Finally, one-way ANOVA indicated no statistical difference in the mean among Zones I–III ($p>0.05$).

Discussion

Our data demonstrate that spheroid culture of hESC-derived ONPs is an advantageous system to create cell aggregates suitable for transplant by micropipette. Spheroids retained 3D form over the culture period. However, cell viability patterns altered over time: younger spheroids contained some apoptotic cells around the edges, and apoptosis increased with time, with apoptotic cells distributed uniformly (in a nonconcentric fashion) in older spheroids. Previous studies that performed TUNEL staining on 3D spheroids mostly demonstrated a concentric configuration of apoptotic cells, with center cell death presumed to occur due to hypoxia and nutrient deprivation in the spheroid core.^{55,56} It is conceivable that the duration of our culture period may not be long enough to show this concentric apoptosis pattern. In addition, the high viability of the spheroids could be due to high biocompatibility of our spheroid-generating platforms. Although cell death was not extensive enough to

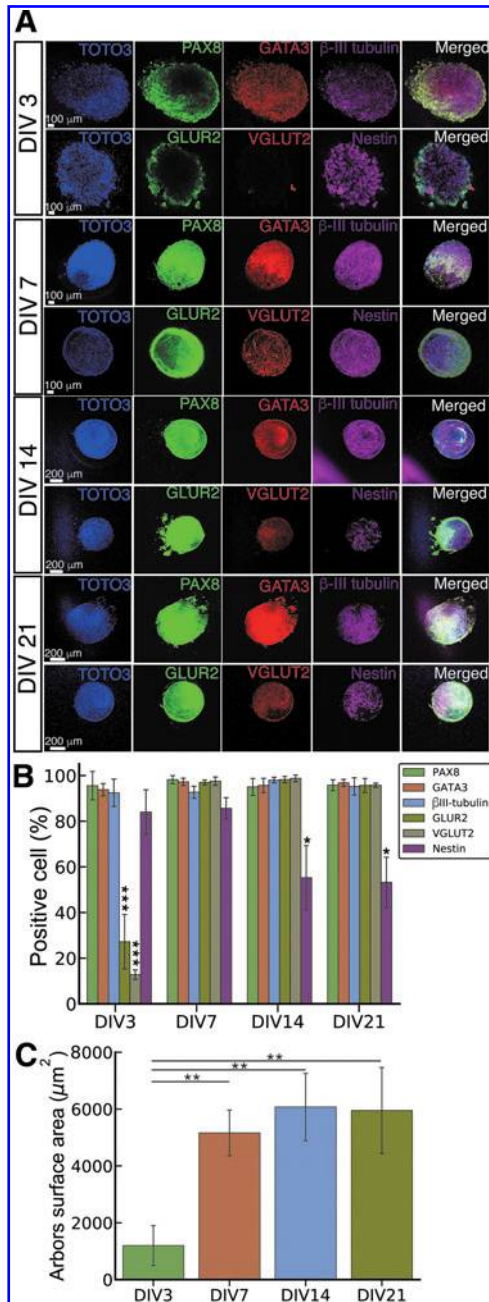


FIG. 4. Immunocytochemical analysis of hESC-derived ONP spheroids. **(A)** Immunocytochemical assessment of ONP spheroids shows expression of the following otic neuronal markers: PAX8, GATA3, β -III tubulin, GLUR2, VGLUT2, and Nestin. Spheroids also show a TOTO-3 iodide nuclear counterstain. Note that stains have been pseudocolored. **(B)** Quantification of positive cells (%) at four different time points (DIV 3, 7, 14, and 21). **(C)** Arborization quantification of hESC-derived ONP spheroids at each time point ($n = 10$ for each time point). *, **, & ***Indicate statistical significance with one-way or two-way ANOVAs with Tukey's *post hoc* test ($p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively). Color images are available online.

destroy the spheroids' 3D configuration, future studies should explore methods to increase cell viability further throughout the spheroids for extended culture periods.

Immunocytochemistry on the 3D spheroid culture showed that component cells expressed markers specific to the ONP

stage (the precursor to SGNs) by DIV 7: β -III tubulin, GLUR2, and VGLUT2. Spheroids also showed continuous high expression of the otic markers GATA3 and PAX8. Our previous study demonstrated that hESC-derived ONPs did not express S-100 (a Schwann cell marker), PAX6 (a marker for the central nervous system), E-cadherin, or myosin VIIA (hair cell markers), indicating guidance toward the otic lineage.⁵ Nestin, the only observed marker to drop significantly in expression over the culture period, is a neural marker found most often in progenitor cells.^{45,57} Its decrease in expression indicates that 3D culture promotes continued ONP differentiation from the progenitor stage. Additionally, our neurite arborization assay shows maximized neurite growth by DIV 7, indicating that spheroids should be cultured until at least DIV 7 before transplant to maximize expression of these specific neuronal progenitor markers.

The whole-cell voltage-/current-clamp recordings showed early signs of functional development in our ONP spheroids. Although spheroids at DIV 5 showed little ion channel activity, by DIV 7 a large voltage-dependent outward current was clearly apparent, indicating functional Kv channel expression. Additionally, the transmembrane potential repolarization following current-induced cell depolarization seen in DIV 7 spheroids may be an early sign of neuronal maturation, although this can be also seen in a variety of cells in animals.⁵⁸ The repolarization was not present in spheroids at DIV 5, demonstrating the need to allow additional time for neuronal development. We acknowledge that the ONP spheroids generated in this study were functionally not fully matured; improved resting membrane potentials (-50 to -60 mV) were found in some ONP cells at DIV 10 or later, but none showed significant inward currents upon cell depolarization. Previous studies that investigated electrophysiological properties of hESC-derived SGNs have shown that cells at least later than DIV 10 showed voltage-activated inward currents and/or action potentials.^{7,47,59,60} We, therefore, speculate that these activities can be promoted by extended culture, which should be explored in future study.

Spheroids seeded in both culture platforms, U-bottom and EZSPHERE, increased in stiffness over time, but not at the same rate. As early as DIV 4, all spheroids had Young's moduli similar to that of mouse brain and were too fragile to hold up to the stress of mock transplantation. By DIV 8, U-bottom spheroids were stiff enough for transplantation, and EZSPHERE spheroids by DIV 11. We conclude that both systems are sufficient to form spheroids robust enough to hold up to transplantation, but U-bottom spheroids would allow us to perform a transplantation procedure more quickly. If, however, large-scale production of spheroids is required, the EZSPHERE platform is preferable, since it does not require time-consuming seeding of individual spheroids.

The increase in elastic modulus over time may be correlated with changing cell morphology within the 3D structure. For instance, cell stiffness and motility are regulated by cytoskeletal structures such as F-actin filaments; it is therefore possible that F-actin filaments on the spheroid surface contribute to the increasing stiffness.^{21,61} Future study can explore this further by cytoskeletal F-actin imaging. An improved understanding of the mechanisms responsible for mechanical changes over time may provide

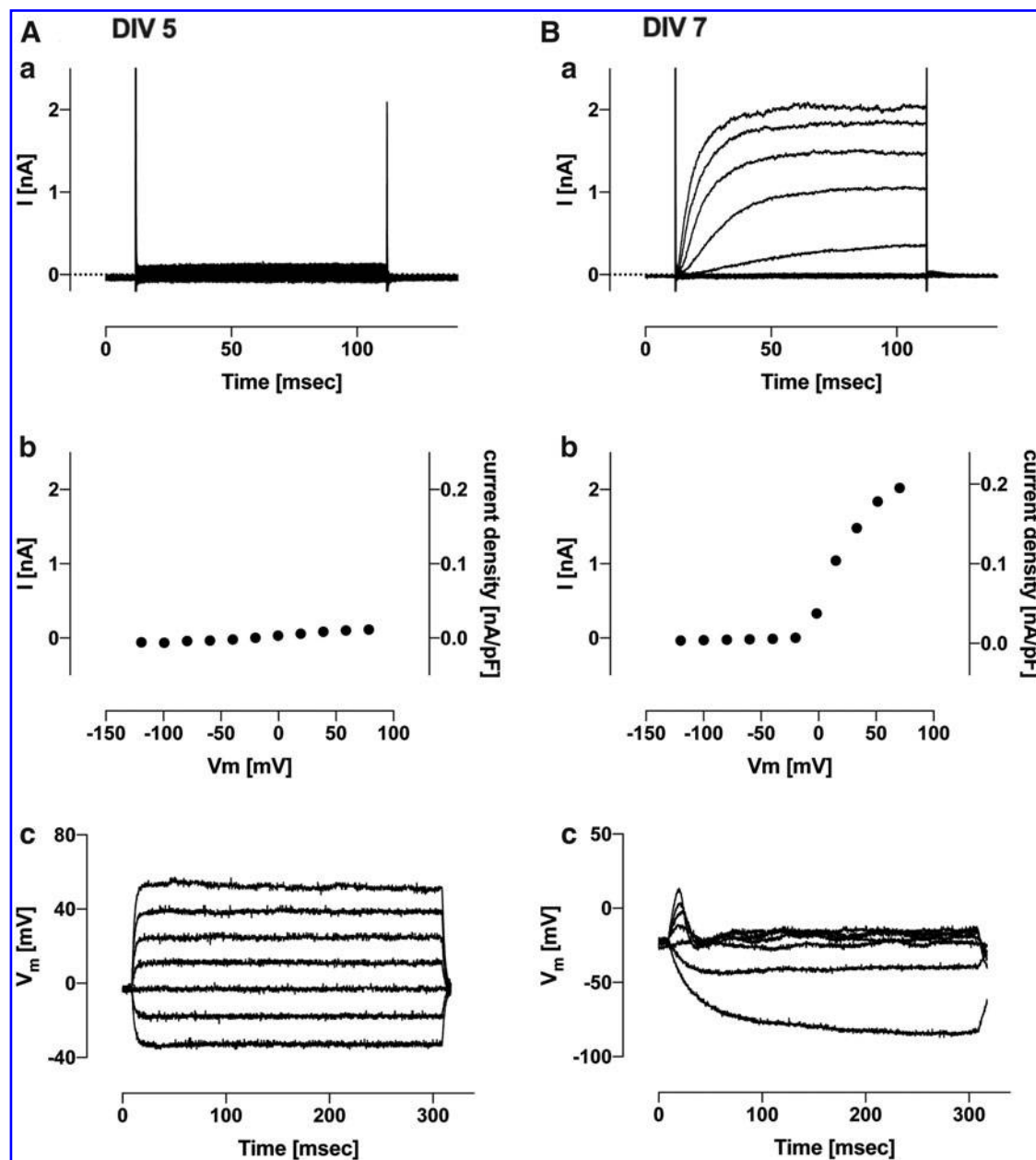


FIG. 5. Electrophysiological responses and properties of hESC-derived ONP spheroids. **(A)** DIV 5 and **(B)** DIV 7 hESC-derived ONP spheroids. **(a)** Whole-cell voltage-clamp recordings of hESC-derived ONP spheroids that were subjected to 100 ms rectangular steps with voltages from -120 mV to $+80$ mV (20 mV step). The holding potential was -60 mV. **(b)** Current/voltage (IV) relations in the hESC-derived ONP spheroids. **(c)** Whole-cell current-clamp recordings of hESC-derived ONP spheroids. The magnitudes of the injected current were from -30 pA to $+60$ pA (15 pA step) and -75 pA to $+150$ pA (37.5 pA step) for **A(c)** and **B(c)**, respectively.

new insight into approaches for chemically targeting cytoskeletal structures that regulate cell stiffness and motility.⁶¹

Our observations of elastic Young's moduli of hESC-derived ONP spheroids were consistent with previously published data using microtweezers or atomic force microscopy.^{21,62} It is worthwhile mentioning that a different Poisson's ratio (e.g., 0.3) would change the estimation of Young's modulus (e.g., increasing by 21%); however, our purpose in this study was to demonstrate relative differences in the Young's moduli rather than absolute values. We used a Poisson's ratio of 0.5 based on a previous study.^{62–65}

Additionally, previous studies indicate that measurements of the elasticity of biological samples by different methods—for example, optical tweezers or rheometers—have quite a wide range of variance.^{62,66,67} Although the variance may arise from differences in cell culture conditions, the particular geometry of the probe used and the amount of mechanical stress put on the cells in each experimental method play a significant role.⁶⁶ Thus, it should be noted that absolute values between experiments are rarely directly comparable.^{66–68}

Our mock transplantation procedure assessed the damage done to ONP spheroids during transfer. All spheroids exhibited

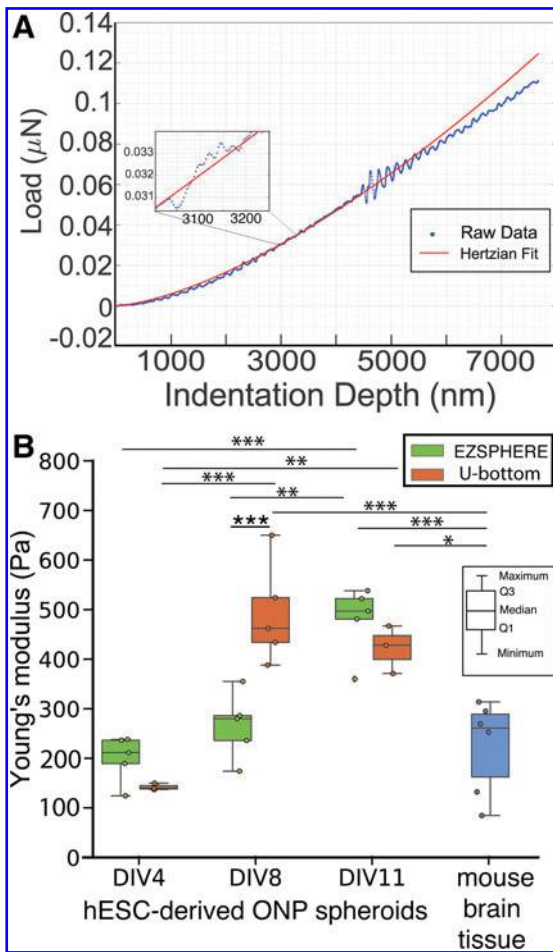


FIG. 6. Mechanical properties of hESC-derived ONP spheroids. (A) An exemplary force curve obtained from nanoindentation testing of an hESC-derived ONP spheroid. The raw data (blue) was fit to the Hertzian curve (red). *Inset:* Magnification of force curve demonstrating the smoothing effect of the Hertzian curve fit to the raw data. (B) Young's modulus of hESC-derived ONP spheroids at three different time points (DIV 4, 8, and 11). Mouse cerebral cortex tissue was also measured as a control. Brain: mouse cerebral cortex tissue. *, **, and *** indicate statistical significance with two-way ANOVA with Tukey's *post hoc* test ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Q1: the first (25th percentile) quartile; Q3: the third (75th percentile) quartile. Each gray box indicates interquartile range (IQR). Maximum whisker: $Q3 + 1.5 \times IQR$; Minimum whisker: $Q1 - 1.5 \times IQR$. Each biological replicate is shown as a circle and represents the average of three technical replicate measurements. For each attribute, Shapiro–Wilk test was performed to confirm a Gaussian distribution before performing two-way ANOVA. Note that outliers were not included for two-way ANOVA (outlier is defined as $>99.7\%$ or $<0.03\%$). Color images are available online.

a proportion of dead surface cells after micropipette manipulation. Smaller micropipettes showed larger variations in proportions of living cells, however, indicating lower reliability for transplantation. Additionally, smaller micropipettes required a vast pressure differential to capture and release spheroids, creating significantly more stress and leading to a greater degree of spheroid deformity. It can therefore be concluded that micropipette tips with sizes similar to the spheroids themselves are

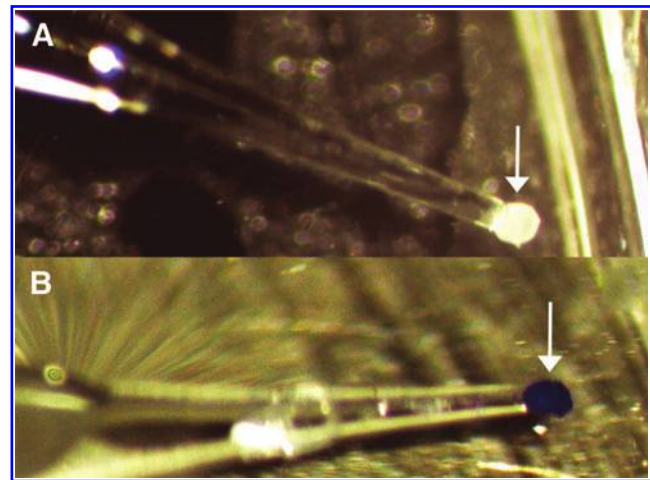


FIG. 7. Micropipette apparatus for simulated transplant of spheroids. Negative pressure holds hESC-derived ONP spheroids at the tip of the glass micropipette (see white arrows for the spheroid location). (A) Unstained spheroid. (B) Spheroid stained with gentian violet dye. In both cases, the spheroid diameter is 240–260 μm and the negative pressure required for spheroid capture was ~ 5 hPa. Color images are available online.

most suitable for use. The Sutter Xenowork system we used in this study has been widely used for somatic cell nuclear transfer and intracytoplasmic sperm injection, but to our knowledge, this is the first study that used glass micropipettes in conjunction with a microinjector for stem cell spheroid transfer.⁶⁹ This work thus constitutes an intermediate step toward the development of *in vivo* transplantation procedures for hESC-derived 3D aggregates.

We found that DIV 7 was the optimal time point for our *in vivo* spheroid transplantation. Neurite arborization reached a maximum at this point and did not statistically differ thereafter. In addition, the elastic properties of our spheroids indicated that U-bottom spheroids were stiff enough to support transplantation after DIV 7 (and EZSPHERE spheroids by DIV 11). Furthermore, using our curve fit equation (Fig. 2C), it took 7 days for our hESC-derived ONP spheroids to approach a diameter of 500–700 μm —the diameter of the round window in the mouse.⁷⁰ Based on these results, we conclude that it is most suitable to transplant our hESC-derived ONP spheroids to the inner ear at or after DIV 7. Our curve-fitted spheroid diameter model (Fig. 2C), in addition to our mock transplant data (Fig. 8), can be used to identify suitable seeding density, culture period, and micropipette size for optimal transplant of hESC-derived ONP spheroids to the inner ear in future studies. This information can be utilized by the auditory research community for further development of improved stem cell replacement therapies.

Several findings of this study warrant further discussion. First, this study solely focused on hESC-derived ONP spheroids. Stem cells can also be obtained from autologous (i.e., mesenchymal stem cells or inner ear stem cells) or allogeneic sources (i.e., mouse embryonic stem cells). The results we reported in this study are only applicable for hPSCs, and further investigations will be needed for other types of cells. Second, while our TUNEL viability assay demonstrated that apoptosis occurred in a nonconcentric

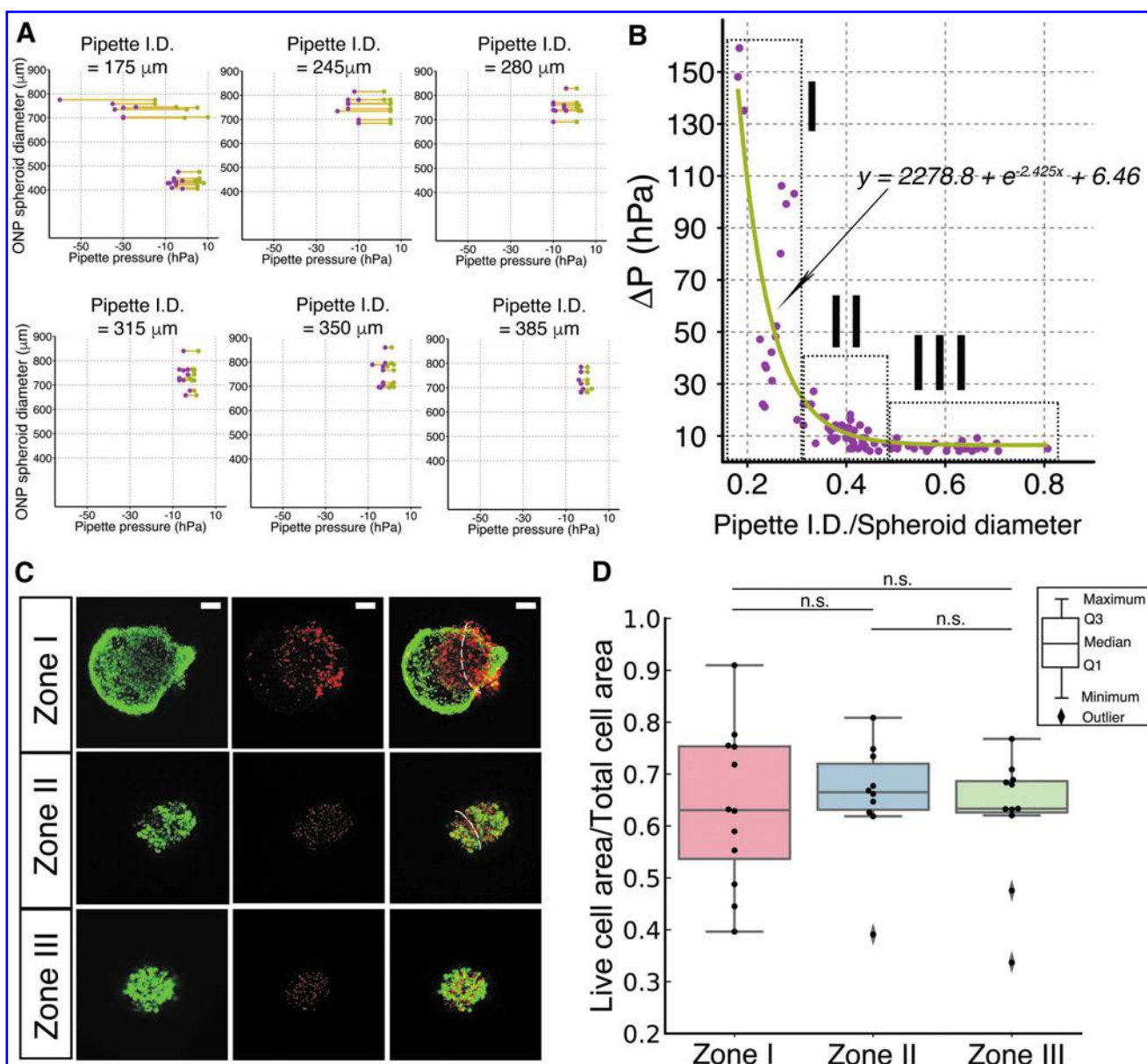


FIG. 8. Simulated transplant procedure for hESC-derived ONP spheroids and analysis of cell survival. **(A)** Pressure differential required to capture (purple) and release (green) individual spheroids, separated by micropipette diameter. I.D.: inner diameter. **(B)** Plot of the change in pressure required to capture and release individual spheroids against the ratio of micropipette inner diameter to spheroid diameter. Green line indicates nonlinear curve-fit. **(C)** Confocal photomicrographs of hESC-derived ONP spheroids after transfer by micropipette with inner diameter size 10–30% (Zone I), 30–50% (Zone II), or 50–85% (Zone III) of the diameter of the transferred spheroids. Green: live cells; red: dead cells. Scale bar = 100 μm . The white dotted line indicates the location of contact between spheroid and pipette. **(D)** Proportion of live cells in surface images of spheroids divided by zone. n.s.: statistically not significant (one-way ANOVA with Tukey's *post hoc* test); Q1: the first (25th percentile) quartile; Q3: the third (75th percentile) quartile. Each gray box indicates interquartile range (IQR). Maximum whisker: $Q3 + 1.5 \times \text{IQR}$; Minimum whisker: $Q1 - 1.5 \times \text{IQR}$. Each biological replicate is shown as a black dot. Outliers are defined as having data belonging to the range $>99.7\%$ or $<0.03\%$ (normal distribution was confirmed with a Shapiro-Wilk normalization test). Color images are available online.

fashion over time, further investigations are needed to evaluate the status of the core of the spheroids and determine whether cell death was due to necrosis or programmed apoptosis. Third, the data on mechanical properties should be viewed with discretion, as testing was performed solely with a nanoindenter; future experiments will use a second method to verify our results.

Taken together, our data describe a comprehensive system for developing hESC-derived ONP spheroids and optimizing their transplant. Our cells self-aggregate into spheroids easily and show the characteristics of neuronal progenitors; these characteristics are most apparent after at least 7 days of spheroid culture. Moreover, our transplantation protocol is successful in transferring these cells as

aggregates with minimal destruction of the spheroids. To the best of our knowledge, this is the first study to investigate the transplantation of 3D cell aggregates—spheroids or organoids—using a micropipette aided by a digital microinjector. Although preliminary, our findings indicate that these hESC-derived ONP spheroids are a favorable candidate for inner ear transplantation therapy to aid in the regeneration of SGNs and mitigate the effect of SNHL.

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Disclosure Statement

This article has not been considered to be published in another journal. The authors have no competing and financial interest.

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Supplementary Material

Supplementary Data
Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

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Supplementary Data

Supplementary Materials and Methods

Image acquisition and quantification of cells

The level of fluorescence in a given region (e.g., nucleus) was determined using the corrected total cell fluorescence (CTCF) based on the following formula:

$$CTCF = Integrated\ Density - (Area\ of\ selected\ cell \times Mean\ fluorescence\ of\ background\ readings)^{S1-S3}$$

TOTO3-iodide-stained cells were counted using the ITCN (image-based tool for counting nuclei) plugin for ImageJ developed by Thomas Kuo and Jiyun Byun at the Center for Bio-image Informatics at the University of California Santa Barbara.^{S4} Images were converted to 8-bit grayscale and inverted before using the ITCN. Cell detection was performed detecting dark peaks with the following parameters: cell width=7, minimum distance=7, threshold=2, mask image: use selected ROI. The number of positively labeled cells was counted in 10 randomly selected fields and was expressed as a percentage of the total number of cells counted. The total number of cells was quantified by TOTO3 iodide nuclear staining.

Mechanical characterization of otic neuronal progenitor spheroids

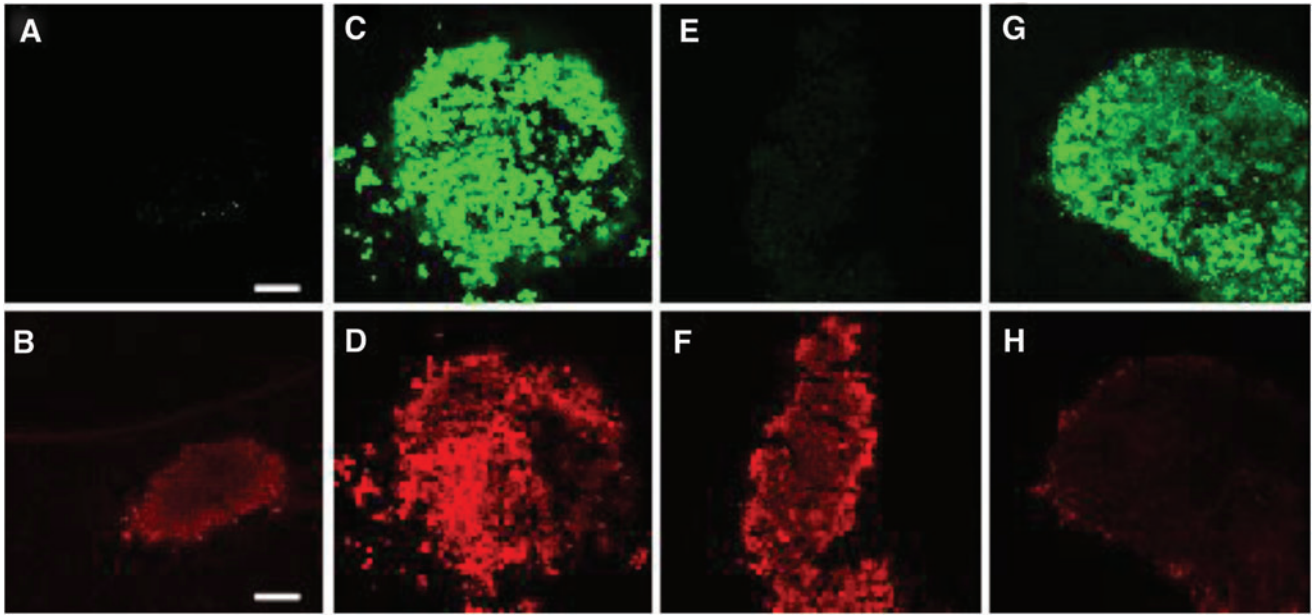
Human embryonic stem cell (ESC)-derived otic neuronal progenitor (ONP) spheroids were allowed to self-assemble using two culture plates: 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (U-bottom) or EZSPHERE®. Each plate promotes the self-assembly of cell spheroids, but the U-bottom culture plate allows for both seeding of individual spheroids and centrifugation after seeding to aid cell clustering. Resulting spheroids from each type of culture plate were tested with an Optics11 Piuma nanoindenter to observe spheroid stiffness. The Piuma nanoindenter was set to a constant displacement rate of 4000 nm/s with a top hold

time of 0.5 s (initial stage) and an indent hold time of 1.0 s (final stage). We chose a cantilever with stiffness of ~0.5 N/m based on the manufacturer's recommendation. The cantilever size was chosen such that the spheroids appeared locally flat to the probe to avoid any artifact from the spheroids' roundness. The estimated tip radius was 27 µm. With regard to indentation depth, the Piuma nanoindenter is designed for surface measurements. A larger indentation may have caused the probe to start detecting the substrate on which the spheroids were resting. In general, the sample must be properly immobilized to avoid any lateral movement during indentation. The sample also has to have an appropriate thickness, typically more than 10 times the desired indentation depth.^{S2} In this way, one can be sure the properties of the substrate will not significantly affect the result of the measurement. Previous studies measuring the elastic Young's modulus of other types of spheroids also kept the depth of indentation below 10 µm to avoid this potential complication.^{S3,S4}

The probe was mounted in the Piuma nanoindenter and the optical signal was calibrated. Manual find-surface approach was used. After each experiment, the load-displacement data were saved in a text file format for postprocessing. For each matrix scan, a comma-separated text file (csv) was saved with a matrix of the Young's modulus per coordinate. Human ESC-derived ONP spheroids at DIV 4, DIV 8, and DIV 11 (at least three biological replicates for each DIV; three technical replicate measurements were averaged to produce one biological replicate) were used. Mouse brain tissue was used as a control. The Institutional Animal Care and Use Committee of Northwestern University Feinberg School of Medicine approved our experiment procedures (IACUC Protocol number: IS00000379), which also met U.S. National Institutes of Health guidelines for animal care and use. A wild-type mouse (C57BL/6 strain) (The Jackson Laboratory, Bar Harbor, ME) was used. Immediately after euthanizing, the mouse brain was harvested and slices were generated at a thickness of 400 µm using a McIlwain™ tissue chopper. Slices were then



SUPPLEMENTARY FIG. S1. Fluorescence images of an hESC-derived ONP spheroid for a secondary antibody control experiment. (A) TOTO3 iodide counterstaining; (B) donkey anti-goat Alexa Fluor 488 (D-anti-Goat-488); (C) donkey anti-mouse Alexa Fluor 594 (D-anti-Ms-594); (D) donkey anti-rabbit Alexa Fluor 405 (D-anti-Rb-405), and a composite image (E). Each sample was treated identically to experimental immunocytochemistry conditions with the omission of primary antibody. Note that images have been pseudocolored. TOTO3, TOTO3 iodide; D, donkey; Ms, mouse; and Rb, rabbit; ONP, otic neuronal progenitor; hESC, human embryonic stem cell.



SUPPLEMENTARY FIG. S2. Positive and negative controls of the Live/Dead Assay performed on GP293 spheroids. *Upper row:* live cells (green); *lower row:* dead cells (red). Killed spheroids were treated with 70% ethanol, and fixed spheroids were treated with 4% paraformaldehyde. (A, B) A killed, unfixed GP293 spheroid. (C, D) A live, unfixed GP293 spheroid. (E, F) A killed, fixed GP293 spheroid. (G, H) A live, fixed GP293 spheroid. Scale bar = 100 μ m.

immobilized on a 10-cm culture dish bathed in neurobasal medium (Thermo Fisher Scientific, Waltham, MA).^{S5,S6}

MATLAB (version 9.5.0 1067069 (R2018b) Update 4; The MathWorks, Inc., Natick, MA) was used to analyze the load–displacement data. The Hertz method was used for the analysis because the use of the loading portion of the load–displacement curve is far more suitable for soft samples such as biological material.^{S7} The Hertz model assumes a linear elastic material response.^{S7} The formula used to calculate the elastic Young’s modulus was as follows:

$$E = \frac{3(1 - \nu^2)P}{4R^{1/2}h^{3/2}}$$

where P is load, E is the elastic Young modulus, h is the cantilever deflection (nm), R is the radius of the spherical indenter tip (μ m), and ν is Poisson’s ratio. MATLAB functions called “*xlsinfo*” and “*xlsread*” were used to extract the load–displacement curve data and then obtain maximum value of load (P) parameters given that we only needed the loading phase for the Hertz method. The data from the first “non-zero” number to the maximum value of load (P) were saved as a variable named “*hloading*.” We used a nonlinear numerical power–law curve fit modulus in MATLAB toolbox called “*curve-fitting toolbox*”. Poisson’s ratio was set to 0.5 based on data in similar studies previously published.^{S5,S8}

Whole-cell patch-clamp recordings

Recording pipettes were pulled from borosilicate glass to achieve initial bath resistances averaging 3–4 M Ω . Recording pipettes were filled with an intracellular solution con-

taining: 140 mM KCl, 2 mM MgCl₂, 10 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA; Thermo Fisher Scientific), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Thermo Fisher Scientific). Cells were bathed in Hank’s Balanced Salt Solution (HBSS; Thermo Fisher Scientific). Pipette offset current was zeroed immediately before the pipette tip contacted the cell membrane. Intracellular pressure was kept at 0 mmHg and voltage/current data were collected by jClamp (SciSoft Company, New Haven, CT). All recordings were acquired at room temperature and waveforms were low-pass filtered with a Bessel filter and 3 dB cutoff frequency of 10 kHz. Command voltages and currents were constant step functions of either 100 (for voltage-clamp recordings) or 300 ms (for current-clamp recordings) duration. Data were not corrected for liquid junction potential that was estimated to be $\sim$ 4 mV.

Otic neuronal progenitor spheroid mock transplantation using a micropipette

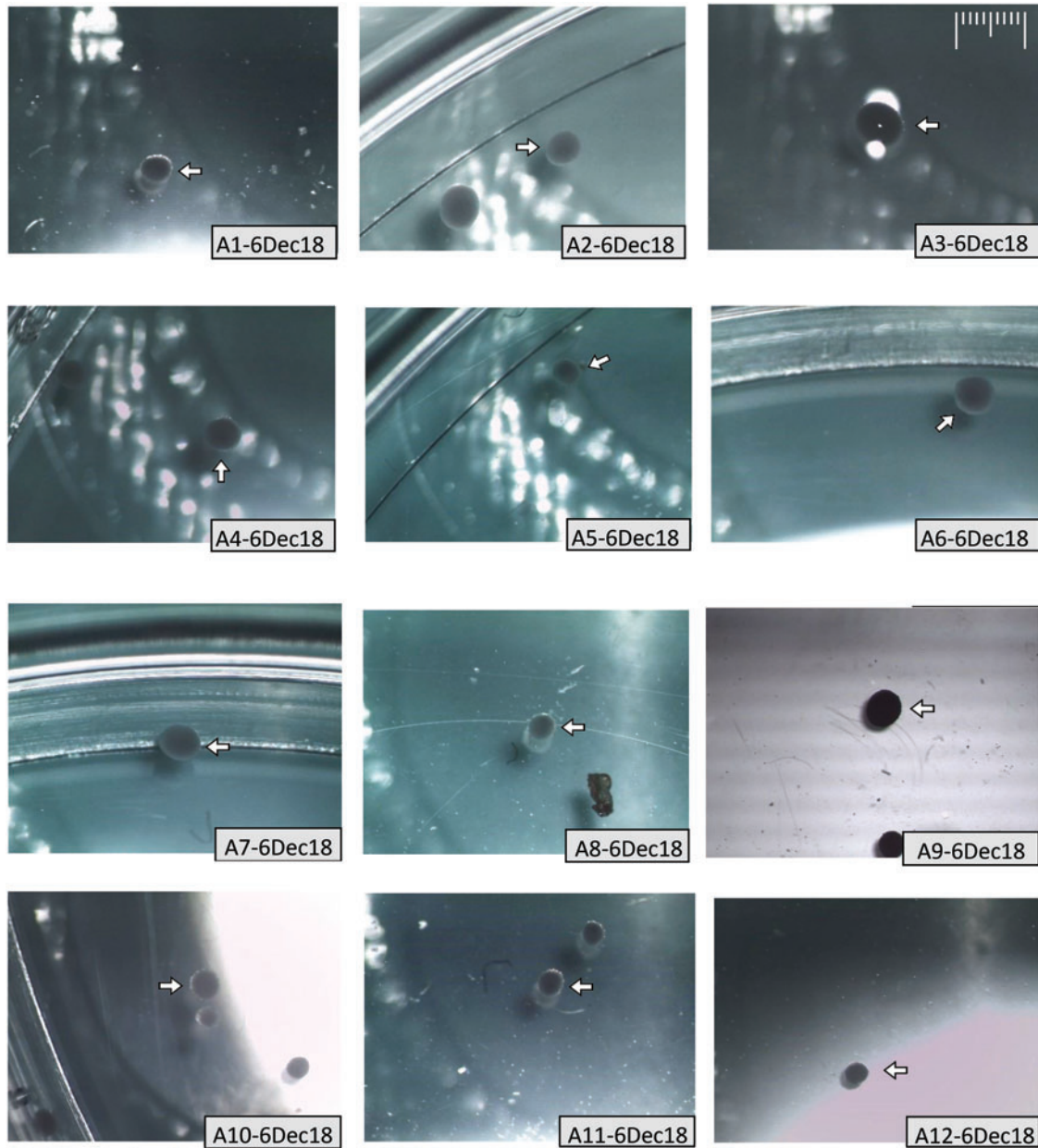
Capillaries, borosilicate glass, thin wall, 5-cm-long, 1.5-mm outer diameter (O.D.), 0.75-mm inner diameter (I.D.), with filament (Catalog #: TW100F-4, World Precision Instrument (WPI), Sarasota, FL) were used. The pipette holds were first pushed together into their locked positions. Borosilicate glass capillaries were placed into the capillary folders and then locked in position. After these processes, a program was selected to run. A heat ramp test was first performed to determine the “RAMP” value for the “heat” parameter. After “RAMP test” mode was selected on the Sutter P-97 puller, a glass was mounted into a designated unit to activate the heating element. Once the pipette was pulled, the number



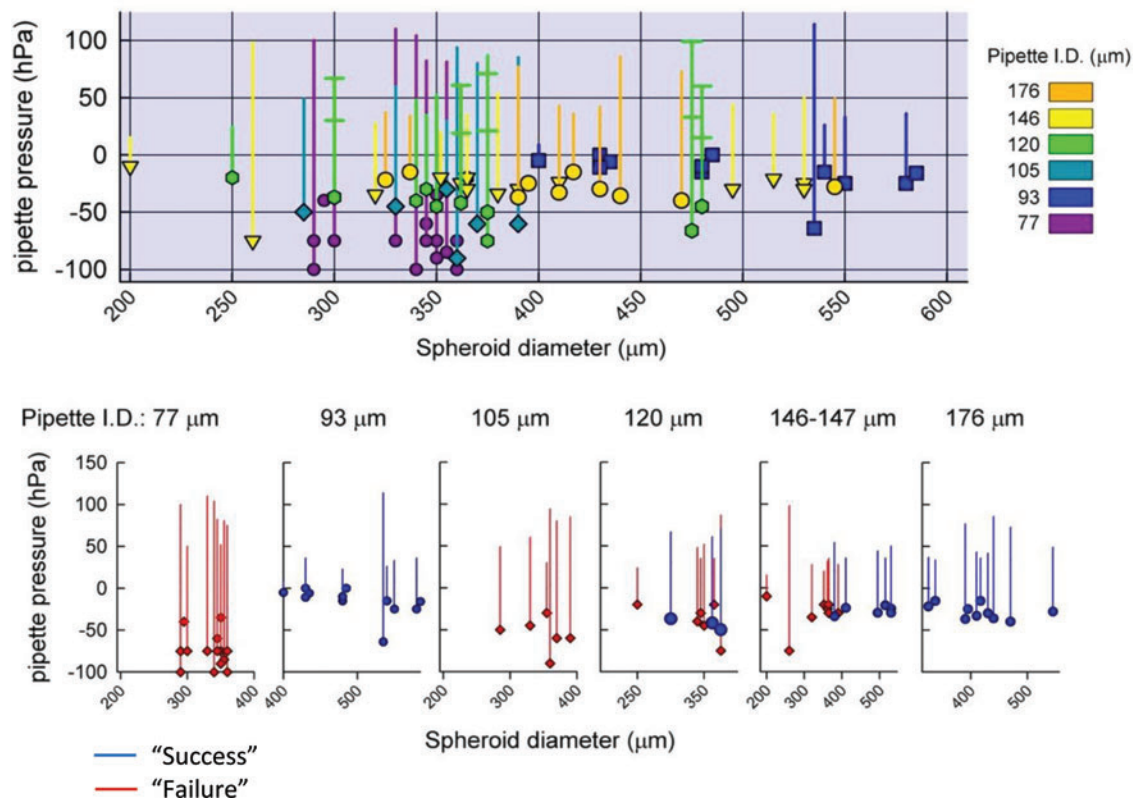
SUPPLEMENTARY FIG. S3. Images of hESC-derived ONP spheroids before transfer. *Arrows* denote transferred spheroids. Each scale interval (*upper right*) indicates 1000 μm . The scintillation observed at spheroid peripheries is caused by light reflection of individual cells. In cases of ovoid spheroids, diameter is estimated by the mean of major and minor axes. These spheroids were successfully transferred with micropipettes with I.D.s covering a range from 10% to 85% of the diameters of the spheroids. I.D., inner diameter.

was recorded. This process was repeated until a similar number was obtained. This number is defined as the “RAMP” value for this particular glass. Average RAMP value was 497 ± 5.46 in our study ($n=47$). Typical parameters on the Sutter P-97 we used in this study were as follows: Heat=

RAMP +25; Pull=0; Velocity=150; Time=100; Pressure=200. Note that our targeted tip size was 20–200 μm in size. The needles were then carefully removed (two pipettes were produced each time) and placed in a plastic pipette storage container with tips down.



SUPPLEMENTARY FIG. S4. Images of hESC-derived ONP spheroids before transfer. *Arrows* denote transferred spheroids. Each scale interval (*upper right*) indicates 1000 μm . In cases of ovoid spheroids, diameter is estimated by the mean of major and minor axes. These spheroids were successfully transferred with micropipettes with I.D.s covering a range from 10% to 85% of the diameters of the spheroids.



SUPPLEMENTARY FIG. S5. A series of quantitative analyses of hESC-derived ONP spheroid transfer experiments. *Top:* ranges of pressure (hPa) used to transfer hESC-derived ONP spheroids in relation to the spheroid diameter (μm) with six different pipette I.D. sizes (μm). Symbol indicates spheroid capture pressure, and vertical line extends to spheroid release pressure. *Bottom:* ranges of spheroid transfer pressures (hPa) in relation to the spheroid diameter with six different pipette I.D. sizes (μm), plotted by individual pipette I.D. Symbol indicates spheroid capture pressure, and vertical line extends to spheroid release pressure. These data indicate that larger pipettes tended result in more successful transfers without destruction of the captured spheroid.

SUPPLEMENTARY TABLE S1. PRIMARY ANTIBODIES USED FOR IMMUNOCYTOCHEMISTRY IN THIS STUDY

Primary antibody	Application	Catalog #	Manufacturer	Location
Anti-PAX8	1:500	ab13611	Abcam	Cambridge, UK
Anti-GATA3	1:500	MAB6330	R&D Systems	Minneapolis, MN
Anti-β-III Tubulin	1:100–1:250	Ab18207	Abcam	Cambridge, UK
anti-GLUR2	1:100	NBP1–46490	Novus Biologicals	Centennial, CO
Anti-Nestin	1:100	ab105389	Abcam	Cambridge, UK
Anti-VGLUT2	1:200	SAB5200276	Sigma-Aldrich	St. Louis, MO

SUPPLEMENTARY TABLE S2. SECONDARY ANTIBODIES USED IN THIS STUDY

Secondary antibody	Application	Catalog #	Manufacturer	Location
Donkey anti-rabbit Alexa Fluor 405	1:250–1:500	ab175649	Abcam	Cambridge, UK
Donkey anti-goat Alexa Fluor 488	1:500	A-11055	Invitrogen	Carlsbad, CA
Donkey anti-mouse Alexa Fluor 594	1:500	A-21203	Invitrogen	Carlsbad, CA

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Full length article

An engineered three-dimensional stem cell niche in the inner ear by applying a nanofibrillar cellulose hydrogel with a sustained-release neurotrophic factor delivery system



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ABSTRACT

Although the application of human embryonic stem cells (hESCs) in stem cell-replacement therapy remains promising, its potential is hindered by a low cell survival rate in post-transplantation within the inner ear. Here, we aim to enhance the *in vitro* and *in vivo* survival rate and neuronal differentiation of otic neuronal progenitors (ONPs) by generating an artificial stem cell niche consisting of three-dimensional (3D) hESC-derived ONP spheroids with a nanofibrillar cellulose hydrogel and a sustained-release brain-derivative neurotrophic factor delivery system. Our results demonstrated that the transplanted hESC-derived ONP spheroids survived and neuronally differentiated into otic neuronal lineages *in vitro* and *in vivo* and also extended neurites toward the bony wall of the cochlea 90 days after the transplantation without the use of immunosuppressant medication. Our data *in vitro* and *in vivo* presented here provide sufficient evidence that we have established a robust, reproducible protocol for *in vivo* transplantation of hESC-derived ONPs to the inner ear. Using our protocol to create an artificial stem cell niche in the inner ear, it is now possible to work on integrating transplanted hESC-derived ONPs further and also to work toward achieving functional auditory neurons generated from hESCs. Our findings suggest that the provision of an artificial stem cell niche can be a future approach to stem cell-replacement therapy for inner-ear regeneration.

Statement of Significance

Inner ear regeneration utilizing human embryonic stem cell-derived otic neuronal progenitors (hESC-derived ONPs) has remarkable potential for treating sensorineural hearing loss. However, the local environment of the inner ear requires a suitable stem cell niche to allow hESC-derived ONP engraftment as well as neuronal differentiation. To overcome this obstacle, we utilized three-dimensional spheroid formation (direct contact), nanofibrillar cellulose hydrogel (extracellular matrix), and a neurotrophic factor delivery system to artificially create a stem cell niche *in vitro* and *in vivo*. Our *in vitro* and *in vivo* data presented here provide sufficient evidence that we have established a robust, reproducible protocol for *in vivo* transplantation of hESC-derived ONPs to the inner ear.

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1. Introduction

Between 25 and 48 million United States (US) residents are afflicted with sensorineural hearing loss (SNHL), usually an irreversible condition [1,2]. SNHL typically results from hair cell (HC) loss, initiating a cascade of trans-synaptic degeneration and the secondary loss of obligatory links to the brain, spiral ganglion

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neurons (SGNs). The degeneration of SGNs follows a distal-to-central progression, with peripheral processes degenerating first [3–5]. This pattern has negative implications for successful hearing restoration through cochlear implants (CIs), the only currently available treatment of SNHL [6,7]. Indeed, improvements in CIs have plateaued in recent years [8,9] due partly to candidates' ears typically having widespread loss of the peripheral processes of SGNs [3,10,11], creating “electrode-neuron gaps” that reduce spatial selectivity. This issue cannot be addressed without correcting this loss.

Stem cell-replacement therapy with human embryonic stem cells (hESCs) has been proposed for restoring damaged HCs and SGNs [12,13]. However, HC replacement is technically challenging, as precise placement in the difficult-to-access scala media is required, the diameter of which is less than 1 mm in humans [14]. Furthermore, HC replacement therapy would still require the restoration of SGN peripheral processes and synaptogenesis. Unfortunately, the most promising report to date of stem cell-based therapy showed neither broad nor cochlear-specific restoration of HC-SGN synapses, nor has this report been successfully replicated [15,16].

Our long-term goal, then, is realizing a next-generation “bio-hybrid” CI that integrates the proven CI approach with human embryonic stem cell-replacement therapy. Such hybrid devices are more practical than stem cell-only approaches, as the latter must overcome the additional challenge of restoring HCs, as mentioned above. Newly regenerated, stem cell-derived SGN neurites can extend between Rosenthal's canal and the scala tympani [4,14,17–19]. By focusing on human stem cell transplantation to the scala tympani, newly regenerated SGNs can bridge electrode-neuron gaps to improve spatial selectivity, preserve tonotopy, and motivate higher CI-electrode densities, which should increase CI performance, user satisfaction, and overall quality of life. It will also broaden the pool of candidates, thus serving a wider patient population [20].

Stem cells normally reside in microenvironments (“niches”) that interact with surrounding cells to regulate and promote proliferation, differentiation, and survival [21,22]. While many questions remain as to which stem cell niche components are fundamental for hESCs to adequately, efficiently differentiate to SGNs, the key players of a stem cell niche generally include cellular components, an extracellular matrix (ECM), and secreted factors [23,24]. In the case of the inner ear, which consists of three primarily fluid-filled chambers—the scala tympani, scala media, and scala vestibuli—all three constituents of a stem cell niche are absent, creating an inhospitable environment for transplanted hESC-derived ONPs [25]. Indeed, previous efforts have been made to transplant hESC-derived ONPs or murine mesenchymal stem cell-derived ONPs into these chambers or even directly into the modiolus where SGNs are housed [26–29]. The survival rate of transplanted cells, however, has been marginally low—on the order of 0.1–1.0% [26,28,30–32]. Therefore, the successful use of stem cell therapy in the inner ear may require the creation of a supportive artificial stem cell niche within the inner ear, though the precise constituents of the normal microenvironment in the inner ear are still unknown.

When transplanted cells are injected into the scala tympani in a dissociated form, the cells first float in the perilymph within the scala tympani and then individually adhere to a layer of squamous epithelium that covers the scala tympani circumferentially [33]. The lack of direct cell contact with other transplanted stem cells, however, can result in insufficient *bona fide* cell-cell adhesion, molecule secretion, and receptor interaction with membrane-bound ligands, leading to poor engraftment and inefficient neuronal differentiation [24]. To circumvent the lack of direct contact in transplanted hESC-derived ONPs in the inner ear, we have generated hESC-derived ONP spheroids [24,34]. We define a

“spheroid” as a self-organizing multicellular structure of stem cells consisting of organ-specific progenitors that allow transplanted cells to maintain direct cell contact among each other, thereby maintaining the secretion of cell-cell adhesion molecules and receptors [35]. We hypothesize that this spheroid structure promotes the survival of cells and facilitates otic neuronal differentiation.

The implantation of a hydrogel ECM can also provide a supportive stem cell niche by integrating a mechanical scaffold with the scala's squamous epithelium [36–38]. ECMs can hold transplanted stem cells in place and localize cell-to-cell signaling. Their integration with spheroids can reduce undesirable agglomeration in addition to insulating cells from injection shearing forces that may lead to apoptosis [39].

Nanofibrillar cellulose (NFC) hydrogel, can mimic native soft tissue ECMs in fiber size and mechanical properties [40–42]. An NFC hydrogel is also injectable and thus capable of delivering cells (such as hESC-derived spheroids) to the desired target. Cellulose is biocompatible, showing moderate foreign body responses, if any, and is safe for stem cell applications, having no known toxicity to hESCs [42–44]. Therefore, we hypothesize that utilizing an NFC hydrogel ECM provides a supportive stem cell niche in the inner ear for hESC-derived ONP spheroids.

Conventional growth factors suffer from fragility and thermo-instability under normal physiological conditions both *in vitro* and *in vivo*, exhibiting short half-lives typically ranging from minutes to hours [45]. So far, this drawback has been addressed by large overprovisions of the requisite growth factor(s) and, *in vitro* cultures, by frequent media changes to maintain a reservoir of bioactive protein in the culture medium. The polyhedrin delivery system (PODS®) is a crystalline growth factor formulation developed to overcome the limitations of standard soluble growth factors. The PODS® technology has adapted viral machinery to encase a chosen growth factor (*i.e.*, BDNF) into polyhedrin protein cases [46–48]. The resultant growth factor co-crystals have slow degradation profiles under physiological conditions and therefore allow the sustained release of embedded bioactive growth factor protein. We focus on brain-derived neurotrophic factor (BDNF), which promotes survival and neuronal differentiation of SGNs *in vivo* and *in vitro* [49–51]. Degradation of the PODS® crystals is facilitated by cell- and protease-mediated processes, allowing the release of physiologically relevant levels of cargo protein (in this case, BDNF) over several weeks. We hypothesize that PODS®-human BDNF (hBDNF) system stably provides BDNF to hESC-derived ONPs, facilitates otic neuronal differentiation, and improves the engraftment of transplanted cells.

The purpose of this study is to create an artificial stem cell niche using hESC-derived ONP spheroids, NFC hydrogel, and a self-sustaining source of BDNF to promote the survival and neuronal differentiation of transplanted hESC-derived ONPs. In this study, we first examined the effects of the NFC hydrogel and PODS®-hBDNF on hESC-derived ONP spheroids *in vitro*, followed by *in vivo* analysis using diphtheria toxin (DT) receptor-positive mice (DTR mice) [52–54]. In the DTR mouse model, the gene for human DT receptor (hDTR) was inserted under the regulation of the promoter for *Pou4f3*, an HC-specific transcription factor [52]. The DTR mouse model serves as a suitable model for our long-term aim (*i.e.*, designing a “biohybrid” CI) because a single DT injection at postnatal day 28 (P28) results in HC wipeout with retention of SGNs [55]. This feature provides us with hESC-derived ONP spheroids to bridge the electrode-neuron gaps between extant SGNs and future biohybrid CI electrodes. We also used a DTR mouse model for this study to create HC damage rather than SGN damage in the bony modiolus because surgical access to the bony modiolus poses significant risks of cerebrospinal fluid leaks and of damaging modiolar blood vessels [14,33], complications that can result in meningitis and inner-ear ischemic stroke.

Toward these ends, we characterized the survival and neuronal differentiation of the embedded hESC-derived ONPs in an artificial stem cell niche using immunocytochemistry and immunohistochemistry along with the viscoelastic and rheological analysis of the NFC hydrogel. Our findings suggest that this combination of factors may enable a future approach to stem cell-replacement therapy for inner-ear regeneration.

2. Materials and methods

2.1. Differentiation of human embryonic stem cells into otic neuronal progenitors

The hESC lines H1, H7, and H9 were used in this study (WiCell, Madison, WI, USA). ONPs were generated based on our previously established protocol [56]. See also the Supplemental Data in detail. A stepwise series of ligands and growth factors was added to a neuronal induction medium (NIM) to promote hESC differentiation toward a late-stage ONP lineage (Fig. 1).

2.2. Three-dimensional spheroid culture with nanofibrillar cellulose hydrogel

The hESC-derived late-stage ONPs were seeded into a 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (#7007; Corning Life Science, Tewksbury, MA, USA) at 50,000 cells per well and centrifuged at 900 rpm for 20 min followed by incubation at 37°C for two days. Late-stage ONP spheroids were also generated by seeding 1 million cells into an EZSPHERE® culture plate (#2,641,701; Nacalai USA, CA, USA) at 37°C for two days. A 96-well Flat-Bottom Clear Plate® (Greiner Bio-One, Nürtingen, Germany) was loaded with GrowDex®-T (#200,103,010; UPM-Kymmene Corporation, Helsinki, Finland) pre-mixed with 800,000 PODS®-hBDNF (#PPH1-50; Cell Guidance Systems, Cambridge, UK) or 20 ng/mL of recombinant human BDNF (RhBDNF) (R&D System, Minneapolis, MN, USA). Spheroids were then carefully transferred from culture plates to a flat-bottom plate with direct injection into the GrowDex®-T solution. GrowDex®-T was diluted to 0.5% (w/v) and 0.375% (w/v) throughout the *in vitro* study based on the manufacturer's instructions. Anionic NFC hydrogel (GrowDex®-T) was kindly provided by the UPM-Kymmene Corporation (Helsinki, Finland). Cellulose kraft pulp was chemically modified and fibrillated to form anionic NFC hydrogels, the diameter of which ranged from 4 to 10 nm and lengths from 500 to 10,000 nm, which were measured using electron microscopy. PODS®-hBDNF was kindly provided by Cell Guidance System (Cambridge, UK). NeuroFluor™ NeuO (#01,801; STEMCELL Technologies, British Columbia, Canada) was used for the detection of live neurons.

2.3. Immunocytochemistry (monolayer culture)

Immunocytochemistry on cells cultured on a two-dimensional (2D) monolayer was performed based as previously described [56]. See also the Supplemental Data for further details. The primary and secondary antibodies used in this study are also listed in Supplemental Tables S1 and S2, respectively. Immunocytochemistry controls were also performed as previously described [57,58]. The primary antibody controls were performed with immunocytochemistry with the primary antibody and hESC-derived ONPs with a second primary antibody to each antigen [57]. Secondary antibody controls were performed each time multiple primary antibodies were used [57]. Labeling controls (detection controls) were performed for a sample from each batch of hESC culture [57]. See Supplemental Fig. S1 for exemplary figures for these control conditions.

2.4. Immunocytochemistry (three-dimensional spheroid culture)

For hESC-derived late-stage ONP spheroids, culture medium was first removed and spheroids were fixed with 4% (w/v) paraformaldehyde for 30 min followed by blocking overnight in a solution composed of 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% Triton X-100 in Dulbecco's Phosphate-Buffered Saline (Corning Life Sciences, Tewksbury, MA, USA). Each sample was incubated for three days at room temperature with the primary antibodies listed in Supplementary Table S3. This was followed by several phosphate-buffered saline (PBS) washes and incubation in the dark for three days at room temperature with Alexa Fluor 488 and 647 (#ab150129, #ab150075; Abcam, Cambridge, MA, USA). Nuclei were stained with 4' 6-diamidino-2-phenylindole (DAPI; #D1306; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min. Images were then assessed using a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). ImageJ ver. 2.0.0-rc-69/1.52p. (National Institutes of Health, Bethesda, MA, USA) was used to quantify the images. Further details on image acquisition and quantification of immuno-positive cells are described in the Supplementary Data.

2.5. Enzyme-linked immunosorbent assay for brain-derived neurotrophic factor

To measure the concentration of BDNF secreted from PODS® crystals, the culture media from both a control and an experimental condition were collected at each time point and immediately stored at –80°C before running an enzyme-linked immunosorbent assay (ELISA). The same method was applied to measure the degradation of RhBDNF. All samples were quantified with a BDNF ELISA kit (#BGK23560; PeproTech, Rocky Hill, NJ, USA), and the results were analyzed with a Synergy HTX Multi-Mode Reader (BioTek, Winocski, VT, USA) at a 450-nm wavelength, as instructed by the manufacturer.

2.6. Rheological characterization of nanofibrillar cellulose hydrogel

Rheological characterization was performed on an MCR302 Rheometer (Anton Paar, Graz, Austria) using a 25-mm diameter plate at 37°C. Each sample was dispensed on the preheated rheometer plate with a gap distance of 1 mm, and excess hydrogel was removed. The testing protocol was then applied to the sample. Each hydrogel sample was used for only one test.

2.7. Quantitative real-time polymerase chain reaction (RT-qPCR)

RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR) were performed using standard techniques. Further details of the method and primers used for RT-qPCR can be found in the Supplementary Data.

2.8. Deafening and genotyping of diphtheria toxin receptor-positive mice

The Institutional Animal Care and Use Committee (IACUC) of the Northwestern University Feinberg School of Medicine approved our experimental procedures (IACUC Protocol number: IS00011516). The protocol was also reviewed and approved by the US Army Animal Care and Use Review Office. Both male ($N = 18$) and female ($N = 19$) DTR mice (3–6 months old) were used as hESC-derived ONP recipients. Ten wild-type (WT) C57/BL6 mice were used as a control. We chose a deaf-animal model in the DTR mouse group and developed a colony from a contribution of DTR mice provided by Dr. W. Edwin Rubel (University of Washington, Seattle, WA, USA). The DTR mice were deafened at a young-mature

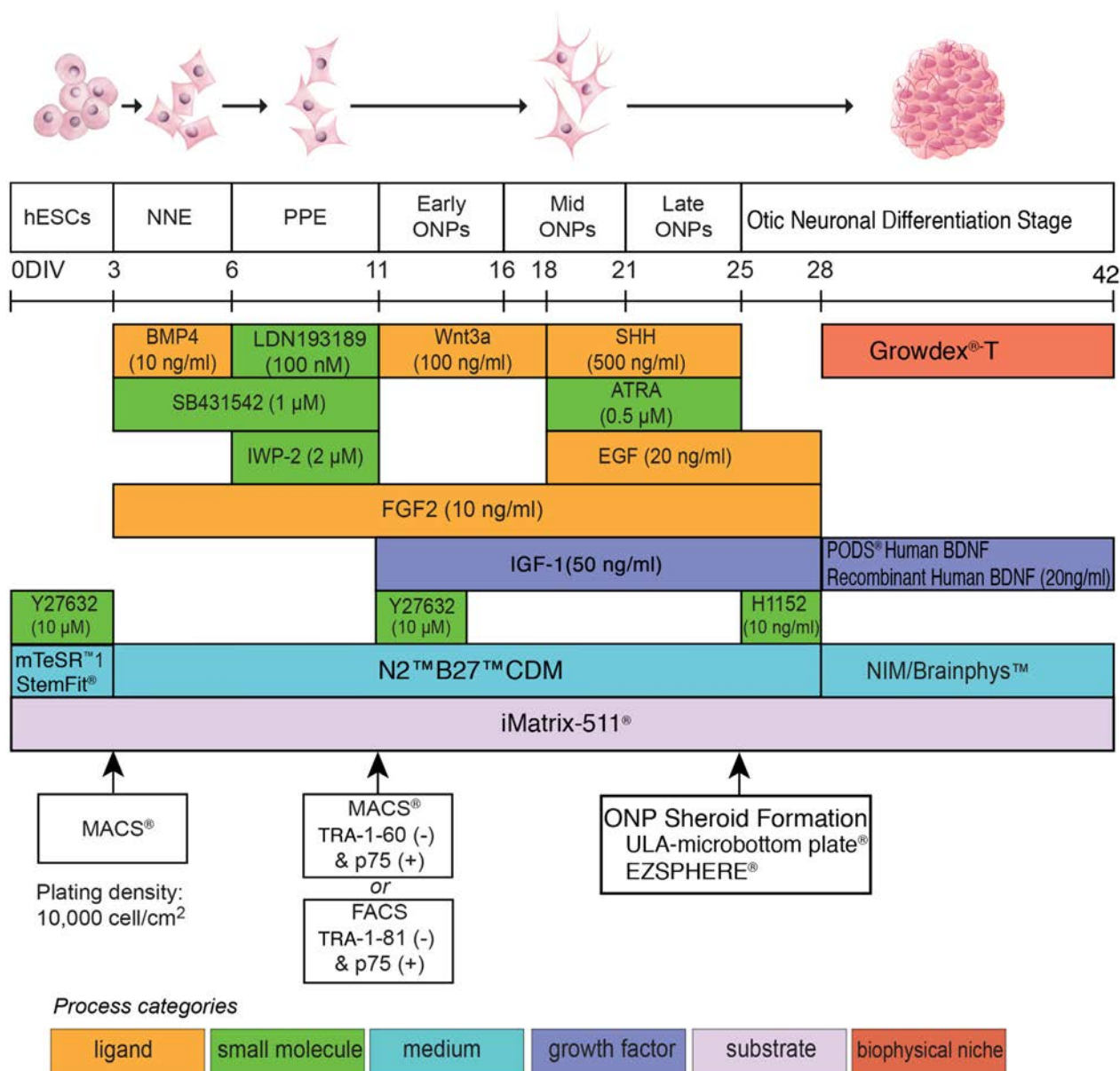


Fig. 1. Illustrative summary of a stepwise treatment protocol for deriving an otic neuronal lineage from undifferentiated H1, H7, and H9 human embryonic stem cells lines. Developmental stages are shown at the top, above a timeline, and key treatments are shown below, using color-coded bars. Abbreviations: D: days; NNE: nonneuronal ectoderm; PPE: preplacodal ectoderm; ONP: otic neuronal progenitor; BMP4: bone morphogenetic protein 4; SHH: Sonic hedgehog; ATRA: all-trans retinoic acid; EGF: epidermal growth factor; BDNF: brain-derived neurotrophic factor; NT3: neurotrophin 3; IGF-1: insulin-like growth factor 1; FGF2: fibroblast growth factor 2; CDM: chemically-defined medium; NIM: neural induction medium; MACS: magnetic-activated cell sorting; FACS: fluorescence-activated cell sorting; p75: low-affinity neurotrophin receptor (p75^{NTR}). This protocol is adapted from Matsuoka et al., 2017 and 2018.

age (P28–P35) using a single intramuscular injection of DT at a dose of 25 ng/g (intramuscular injection), causing HC degeneration [53,54,59]. For identification of DTR mice (*Pou4f3*^{DTR/+}) and WT mice (*Pou4f3*^{+/+}), a tail biopsy was collected and DNA was extracted using a DNA blood and tissue kit (Qiagen, Germantown, MD, USA). The target allele was amplified using a Mastercycler® nexus (Eppendorf, Hamburg, Germany) and the primer (0.4 μM) listed in the Supplemental Data. For further details, see the detailed description of PCR in the Supplemental Data.

2.9. Auditory brain stem response

Hearing loss induced by DT injection was confirmed by auditory brain stem response (ABR) recordings before and after injection. DTR mice were anesthetized with 67 mg/kg of ketamine and 3.3 mg/kg of xylazine (intraperitoneal injection with additional

dosing of ketamine as needed). Both tone-burst and click-evoked ABRs were used to assess the sensitivity of the nerve to acoustic stimuli to confirm the efficacy of the DTR mouse model. Both stimulus control and averaging were performed using TestPoint (Cape Town, South Africa) as well as SmartEP (Intelligent Hearing Systems, Miami, FL, USA). A detailed description of the ABR parameters and settings is provided in the Supplemental Data. We performed tone burst-evoked ABR on both three DTR mice and four WT mice to thoroughly test the frequency-specific hearing threshold. Click-evoked ABR was performed on the remaining mice.

2.10. Transplantation of human embryonic stem cell-derived otic neuronal progenitors

Human ESC-derived ONPs were transplanted in left cochleae. Right cochleae were used as a control condition. Meloxicam

(2 mg/kg, subcutaneous injection) was given prior to surgery to minimize respiratory distress; follow-up doses (1 mg/kg/day) were given for three days. Anesthesia was induced with isoflurane at 3–4% (reduced to 1–2% after induction) and was delivered with oxygen (0.3 L/min) and nitrous oxide (0.25 L/min) gas. The animals' heads were secured to a custom head-holder that delivered the anesthetic gas. Body temperature was maintained by a circulating water pad, and insensate fluid loss was replaced at 0.1 mL/10 g (body weight). A post-auricular incision was made, and bone (caudal to the stylomastoid foramen) was removed with a surgical drill to reveal the round window. The round window membrane was then excised using a 30–33 G needle. Dissociated hESC-derived ONPs (250,000 cells) or hESC-derived ONP spheroids (five spheroids, each containing 50,000 ONPs) were delivered to the scala tympani by a glass micropipette while manipulating the pipette's pressure with a Xenoworks Digital Injector (Sutter Instrument, Navato, CA, USA). The pressure range used for a spheroid transplantation was between -1 hPa and $+5$ hPa. Two μ L of 1% GrowDex[®]-T with 800,000 of PODS[®]-hBDNF were delivered using a 10- μ L Hamilton syringe. After the transplantation was completed, the round window was covered by a small piece of fascia and secured with Vet Bond[™] adhesive (3 M, Minneapolis, MN, USA). Muscle and skin were closed in layers, and the animal was allowed to recover with thermal therapy. The animal was kept for 90 days, allowing engraftment and neuronal differentiation in the inner ear. No immunosuppressant was administered postoperatively [26,30].

2.11. Immunohistochemistry

After completion of the post-implantation survival period, each animal was euthanized, the cochlea was dissected, and the specimen was sectioned into 10- μ m slices on a Leica CM3050 S cryostat (Leica Inc., Nussloch, Germany). Immunohistochemistry was performed using standard techniques with a mouse anti-human nuclear antibody (AHNA) (1:100, STEM101, Takara Bio, Tokyo, Japan) and a rabbit anti- β -III tubulin antibody (1:500, Abcam, Cambridge, MA, USA). See further details on this process in the Supplementary Data. Both positive and negative control conditions were obtained to ascertain the specificities of AHNA (Supplementary Fig. S2). Laser scanning confocal imagery was performed with a Leica TCS SP5 microscope (Leica Inc., Nussloch, Germany). Whole-mount HC staining was also performed on a WT mice, as well as DT-injected DTR mice, and non-DT-injected DTR mice. Further details can be found in the Supplementary Data.

2.12. Neurite-bearing, neurite growth, and neurite arborization assays

The pan-neuronal marker β -III tubulin was used to visualize neurite-bearing cells, measure neurite length, and calculate neurite arborization area. The analysis was performed by examining an individual confocal optical Z-stack section through the entire extent of each spheroid at the observation time point using ImageJ ver. 2.0.0-rc-69/1.52p (National Institute of Health, Bethesda, MD, USA) [60]. Further details can be found in the Supplementary Data.

2.13. Quantification of the surviving anti-human nuclear antibody-positive cells in the inner ear

The total profile number was calculated by the counting profiles of the AHNA⁺ cells [61,62]. Only cells with the confirmation of AHNA-positive immunostaining were counted as surviving ONPs. The total profile number of the AHNA-positive cells was determined for each of the five sections of each animal in four anatomic subdivisions of the cochlea: basal turn, mid-turn, apical turn, and modiolus [63]. A more detailed description of the quantification,

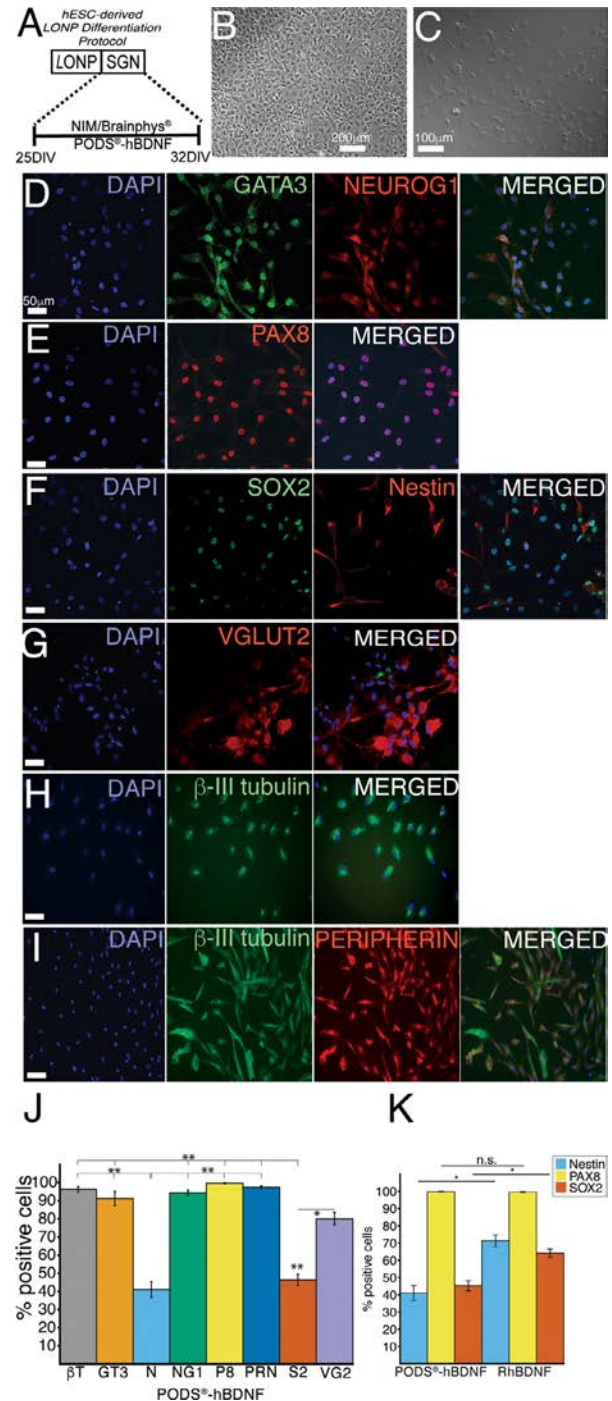


Fig. 2. Assessment of induction of an otic neuronal lineage from late-stage ONPs. (A): A stepwise treatment for an otic neuronal lineage induction. On day 25, PODS[®]-hBDNF treatment was started in NIM for 7 days. D: days. (B): Phase-contrast photomicrographs of hESC-derived late-stage ONPs. (C): Phase-contrast photomicrographs of hESC-derived ONPs treated with PODS[®]-hBDNF for seven days. (D–I): Immunocytochemistry of hESC-derived late-stage ONPs treated with PODS[®]-hBDNF in NIM/Brainphys[™] shows expression of various otic neuronal markers: GATA3, NEUROG1, PAX8, SOX2, nestin, VGLUT2, β -III tubulin, and peripherin. (J): Quantification of the otic neuronal markers for % positivity ($n = 3$) on hESC-derived late-stage ONPs treated with PODS[®]-hBDNF for seven days. BT: β -III tubulin; GT3: GATA3; N: nestin; NG1: neurogenin 1; P8: PAX8; PRN: peripherin; S2: SOX2; and VG2: VGLUT2. (K): Quantification of % positive staining for nestin, PAX8, and SOX2 on PODS[®]-hBDNF and RhBDNF treated cells. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA with Tukey's *post-hoc* test. n.s.: not statistically significant.

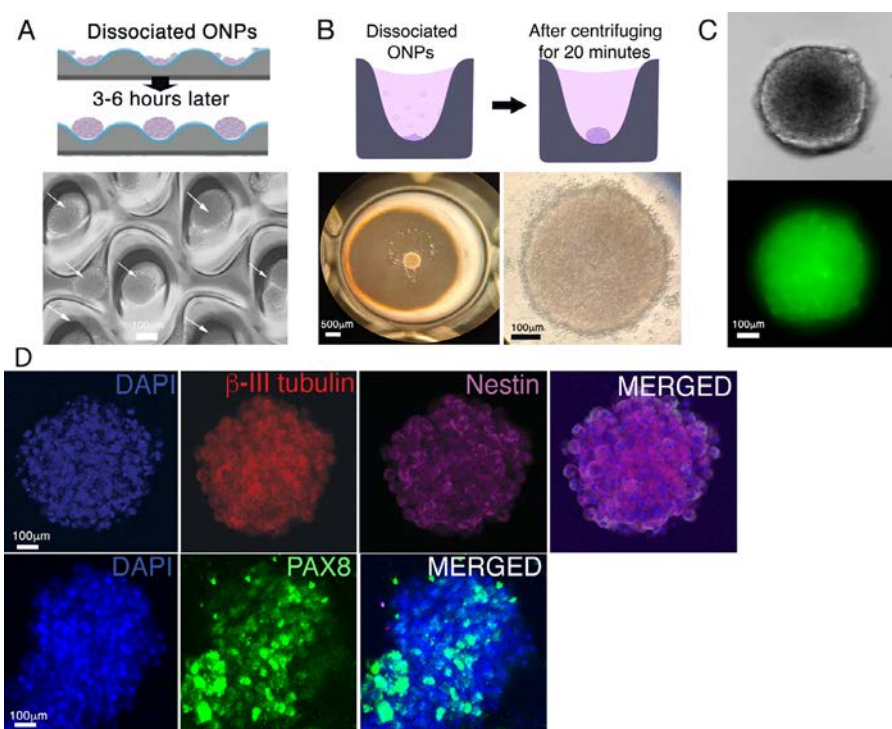


Fig. 3. Generation of hESC-derived ONP spheroids. (A): A schematic diagram of forming hESC-derived late-stage ONP spheroids using an EZSHPERE® plate (upper row) and a phase-contrast photomicrograph of individual spheroids (see white arrow) within the plate (bottom row). (B): A schematic diagram of forming hESC-derived ONP spheroids using a 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (upper row) and a low-power (left bottom row) and high-power (right bottom row) phase-contrast photomicrograph of a spheroid within the plate. (C): A phase-contrast (upper row) and an epifluorescence (bottom row) photomicrographic image of a hESC-derived ONP spheroid stained with NeuroFluor™ NeuO. (D): Immunocytochemistry on a hESC-derived ONP spheroid that was cultured for seven days with 800,000 of PODS®-hBDNF. The spheroid was stained for β -III tubulin, nestin (upper row), and PAX8 (lower row). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including Abercrombie's method of estimating the number of cells from profile counts, can be found in the Supplementary Data [61].

2.14. Calcofluor white staining for nanofibrillar cellulose hydrogels

The presence of NFC in a transplanted cochlea was evaluated with Calcofluor White staining (Sigma-Aldrich, St. Louis, MO, USA). Calcofluor white is a non-specific fluorochrome that binds to cellulose and chitin in cell walls [64]. Three DTR mice transplanted with GrowDex-T and PODS® were used. Due to fluorescence crossover between Calcofluor White and DAPI, no counterstain was used. Further details can be found in the Supplementary Data.

2.15. Hematoxylin and eosin (H&E) histology

Hematoxylin and eosin (H&E) staining was performed on transplanted DTR mouse cochleae to examine the extent of tissue response from xenograft transplantation. Further details of the H&E staining method can be found in the Supplementary Data.

2.16. Detection of major histocompatibility complex class I and class II antigens by flow cytometry

Flow cytometry assays were performed on both hESCs (H9), hESC-derived ONP spheroids, and human embryonic kidney cells (HEK 293 cells: control) using a BD LSRFortessa™ (BD Biosciences, San Jose, CA, USA) using standard protocol. Major histocompatibility complex (MHC) class I and class II antigens were used as transporters because they play critical roles in immune rejection in xenograft [65]. Further details of this method are outlined in the Supplementary Data.

2.17. Statistical analysis

When appropriate, and as indicated in each figure, statistical analysis was performed. Three biological and technical replicates were obtained and used for statistical analysis unless otherwise noted [66,67]. Experimental values are typically expressed as means $\pm$ standard error. All statistical analyses were performed with R (version 3.5.1. 2018-07-02, Vienna, Austria) [68]. Normal distributions were assumed. An analysis of variance (one-way) with Bonferroni-corrected *post hoc* t-tests or two-tailed t-tests was performed. P values smaller than 0.05 were considered statistically significant.

3. Results

3.1. Neuronal differentiation of human embryonic stem cell-derived otic neuronal progenitors with the polyhedrin delivery system

The human ESCs H1, H7, and H9 were sequentially differentiated into late-stage ONPs using the protocol outlined in Fig. 1 and then treated with 800,000 of PODS®-hBDNF for seven days in NIM or BrainPhys™ (Fig. 2A). Compared to late-stage ONPs (Fig. 2B), the treated cells presented more bipolar-shaped neurites, which is consistent with the morphology of human SGNs (Fig. 2C) [69]. After seven days of treatment, late-stage ONPs highly expressed GATA3 (an otic lineage marker) [70,71], Neurogenin-1 (NeuroG1, an otic lineage marker) [72,73], PAX8 (an otic lineage marker; more specifically marking the otic placode and otic vesicle) [74,75], SOX2 (a neuronal progenitor marker) [70], nestin (a neuronal progenitor marker) [70], vesicular glutamate transporter-2 (VGLUT2, a glutamatergic neuronal marker) [76], β -III tubulin (a pan-neuronal

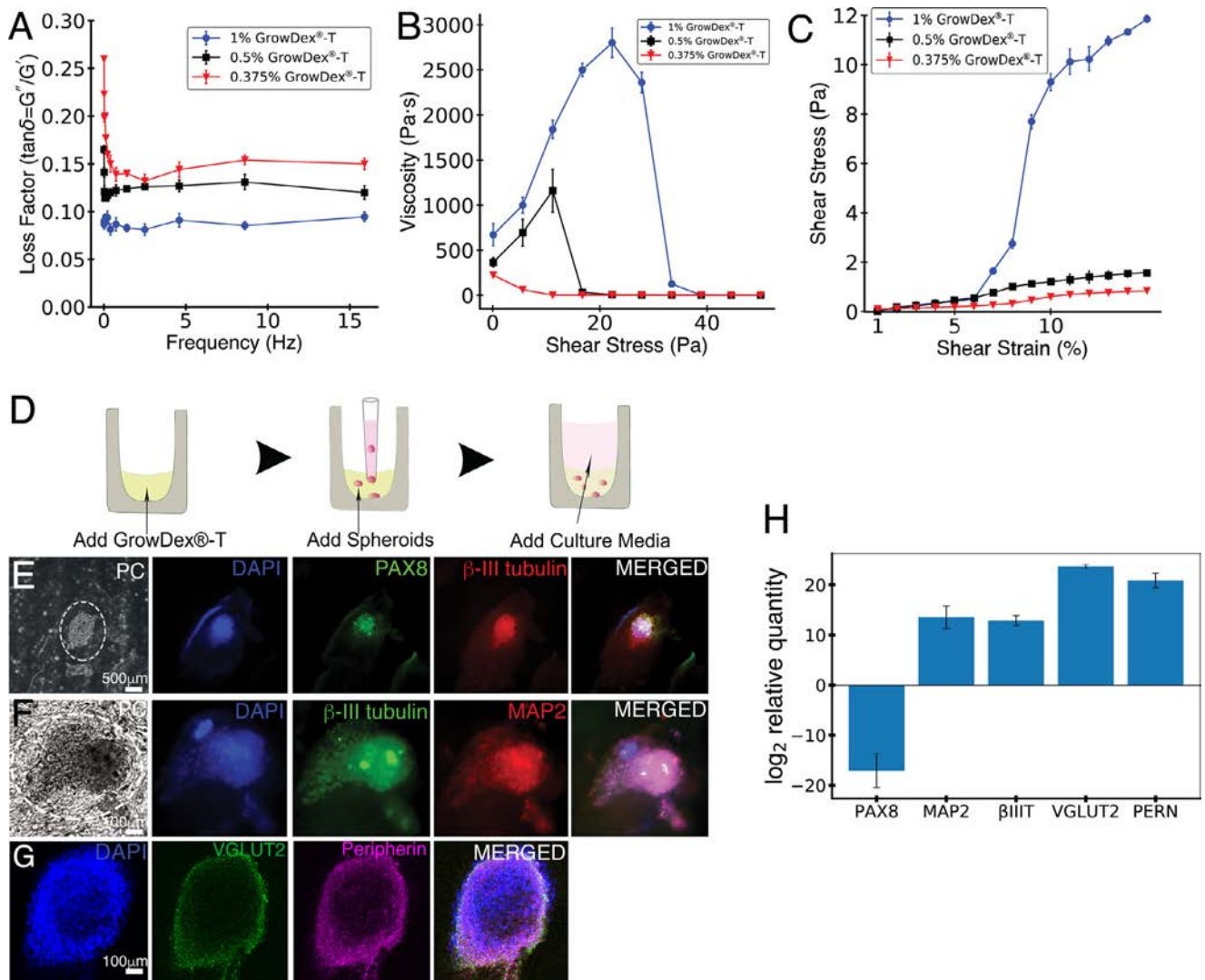


Fig. 4. Rheological characterization of GrowDex®-T and hESC-derived ONP spheroids cultured with GrowDex®-T. **(A):** Frequency sweep (viscoelasticity) of three different concentrations (1%, 0.5%, and 0.375%) of GrowDex®-T ($n = 3$). **(B):** Influence of shear stress on the viscosity of three different concentrations (1%, 0.5%, and 0.375%) of GrowDex®-T ($n = 3$). **(C):** Strain sweep (stress-strain curve) of three different concentrations (1%, 0.5% and 0.375%) of GrowDex®-T. Shear modulus of 1%, 0.5% and 0.375% GrowDex®-T is plotted as a function of shear strain ($n = 3$). **(D):** Schematic figure of hESC-derived ONP spheroids cultured in GrowDex®-T. **(E–G):** Neuronal marker expressions of PAX8, β -III tubulin, MAP2, VGLUT2, peripherin and phase-contrast of hESC-derived ONP spheroids cultured with 0.375% GrowDex®-T. **(H):** Quantitative real-time PCR (qRT-PCR) on hESC-derived ONP spheroids cultured with 0.375% GrowDex®-T and 800,000 of PODS®-hBDNF relative to hESC-derived ONP spheroids cultured in BrainPhys™ with 20 ng/mL of recombinant BDNF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

marker) [77,78], and peripherin (a peripheral sensory neuron marker) [79] (Fig. 2D–I). PODS®-hBDNF-treated ONPs did not express E-cadherin (ECAD, an otic epithelial marker) [80], PAX6 (a central nervous system neuronal marker) [81], nor SOX10 (a glial marker) [82] (data not shown), suggesting that the cells differentiated toward otic neuronal lineage instead of an otic-epithelial lineages, central nervous system neuronal lineages, or glial lineages.

With PODS®-hBDNF treatment of the late-stage ONPs, the proportion of nestin and SOX2 positive cells were expressed significantly less than other markers, suggesting that the treatment facilitated otic neuronal differentiation (Fig. 2J). In the quantification of PAX8, nestin and SOX2-positive cells, the PODS® treatment significantly decreased the positivity of nestin (from $71.3\% \pm 3.42$ to $41.0\% \pm 4.35$) and that of SOX2 (from $64.2\% \pm 2.35$ to $45.2\% \pm 2.90$) compared to RhBDNF treatment, suggesting that PODS® treatment more effectively differentiated the late-stage ONPs into otic neuronal lineages (Fig. 2K). However, there were no statistically signifi-

cant differences in the expression of PAX8 between RhBDNF ($99.6\% \pm 0.294$) and PODS®-hBDNF treatment ($99.8\% \pm 0.222$) (Fig. 2K).

3.2. The generation of human embryonic stem cell-derived otic neuronal progenitor spheroids

The hESC-derived ONP spheroids were generated from dissociated late-stage ONPs using an EZSPHERE® or 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (Fig. 3A–B). A live neuron assay was performed to evaluate the viability of an hESC-derived ONP spheroid cultured in NIM/BrainPhys™ after seven days. An hESC-derived ONP spheroid was labeled with $0.25 \mu\text{M}$ of NeuroFluor™ NeuO and incubated for one hour (shown in green in Fig. 3C), demonstrating that the spheroid was β -III tubulin-positive [83]. The control condition can be found in Supplementary Fig. S3. A 3D immunocytochemical analysis also indicated that hESC-derived ONP spheroids expressed the positivity of PAX8, β -III tubulin, and nestin when cultured in NIM/BrainPhys™ for seven

days (Fig. 3D), suggesting that the spheroids were differentiating into otic neuronal lineages.

3.3. Rheological and viscoelastic properties of nanofibrillar cellulose hydrogel

Before performing a 3D culture of hESC-derived ONP spheroids with NFC hydrogel, the mechanical properties of the hydrogel were first rheologically characterized by frequency sweep testing (Fig. 4A). The frequency sweep in Fig. 4A shows the previously reported characteristic behavior of NFC hydrogel: The loss factor ($\tan \delta = G''/G'$) was much lower than 1 [40]. This finding demonstrates its gel-like structure. This behavior is also nearly independent of the frequency. In addition, 0.375% GrowDex®-T showed a higher loss factor ($\tan \delta = G''/G'$) than both 0.5% and 1% GrowDex®-T. Viscosity over shear stress was also measured to optimize the concentration suitable for the injection of GrowDex®-T, and 1% GrowDex®-T showed higher viscosity than both 0.5% and 0.375% GrowDex®-T at lower shear stresses (Fig. 4B). At high stress levels (>20 Pa, applicable to injections), it also exhibited more fluid-like behavior (Fig. 4B). Shear moduli (G') of different concentrations of GrowDex®-T were estimated to determine their rigidities. In Fig. 4C, 1% GrowDex®-T demonstrates a disproportionally higher shear modulus than both 0.5% and 0.375% GrowDex®-T above a 7% shear strain.

3.4. In vitro culture of human embryonic stem cell-derived otic neuronal progenitor spheroids with nanofibrillar cellulose hydrogel

After the rheological characterization, we cultured hESC-derived ONP spheroids with 0.375% GrowDex®-T for seven days. Fig. 4D shows a schematic diagram of this part of the experiment. Three-dimensional immunocytochemistry was then performed to assess the positivity of PAX8, β -III tubulin, MAP2 (a neuronal cytoskeleton marker) [84,85], VGLUT2, and peripherin to show that hESC-derived ONP spheroids were expressing otic neuronal signals (Fig. 4E–G).

3.5. Quantitative real-time polymerase chain reaction on human embryonic stem cell-derived otic neuronal progenitor spheroids with nanofibrillar cellulose hydrogel

Fig. 4H demonstrates that qRT-PCR on hESC-derived ONPs spheroids cultured in 0.375% GrowDex®-T and 800 K of PODS®-hBDNF for seven days showed more down-regulation of PAX8 and up-regulation of MAP2, VGLUT2, peripherin, and β -III-tubulin than hESC-derived ONPs spheroids cultured in BrainPhys™ with 20 ng/mL RhBDNF for seven days.

3.6. Enzyme-linked immunosorbent assay for brain-derived neurotrophic factor

Before the use of PODS®-hBDNF with our ONP spheroid culture, we quantified the release kinetics of PODS®-hBDNF using ELISA. The concentration we detected from the supernatant of NIM containing 800,000 of PODS®-hBDNF showed steady zero-order release kinetics of hBDNF from PODS®-hBDNF with approximately 25–50 pg/mL per day (Fig. 5A–B). Although PODS®-hBDNF crystals were encased in different concentrations of GrowDex®-T (i.e., 0.5% vs. 0.375%), both groups showed similar release profiles with a total amount of approximately 300 pg/mL over seven days (Fig. 5A–B). On the contrary, a one-time dosage of 20 ng/mL of RhBDNF provided a sufficient amount of hBDNF on the first day, but after three days, the active hBDNF left in both the culture media had less than 100 pg/mL (Supplementary Fig. S4). Finally, we evaluated endogenously secreted hBDNF from hESC-derived

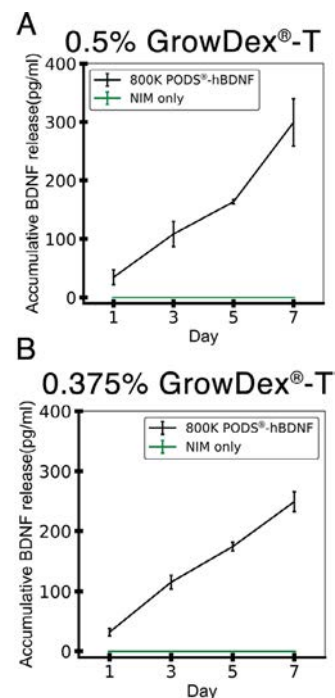


Fig. 5. Accumulative release kinetics of BDNF from PODS®-hBDNF and from a hESC-derived ONP spheroid cultured in NIM. (A): BDNF release profile of 800,000 PODS®-hBDNF in 0.5% GrowDex®-T over seven days. (B): BDNF release profile of 800,000 PODS®-hBDNF in 0.375% GrowDex®-T over seven days. In both (A) and (B), accumulative release kinetic of BDNF from a hESC-derived ONP spheroid cultured in NIM is also plotted in a green line as a control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ONP spheroids. An ONP spheroid (with cell density of 50,000 cells/spheroid) was cultured in NIM without PODS®-hBDNF. There was virtually no BDNF detected in this NIM-only condition with the ELISA spheroids (shown in green in Fig. 5A–B).

3.7. In vitro culture of human embryonic stem cell-derived otic neuronal progenitor spheroids with nanofibrillar cellulose hydrogel and a polyhedrin delivery system

The hESC-derived ONP spheroids were further cultured with GrowDex®-T and PODS®-hBDNF for seven days. The schematic diagram in Fig. 6A shows this part of our experiment. Phase-contrasted images of hESC-derived ONP spheroids cultured in GrowDex®-T and PODS®-hBDNF demonstrate that the integrity of the spheroids tended to decline more with GrowDex®-T and PODS®-hBDNF than with only GrowDex®-T over 48–72 h (Fig. 6B(a–c) vs. Fig. 6B(e–f)). The image also shows that the cells in the spheroids may have been migrating toward PODS®-hBDNF crystals, suggesting that a BDNF gradient may be attracting hESC-derived ONPs in a chemotactic fashion (Fig. 6B(f), white arrow). Fig. 6C–F and Fig. 7A–D show the immunocytochemistry of hESC-derived ONP spheroids cultured with 0.5% and 0.375% GrowDex®-T, respectively, for seven days. As seen in Fig. 6B, hESC-derived ONP spheroids maintained the integrity of their spheroidal shapes better when they were cultured without GrowDex®-T or PODS®-hBDNF (Fig. 6C).

The quantification of PAX8 expression in immunocytochemistry demonstrates the advantages of GrowDex®-T and PODS®-hBDNF in enabling otic neuronal differentiation. We chose PAX8 expression to gauge the degree of otic neuronal differentiation, as PAX8 expression is known to peak at the otic placode or otocyst stages (corresponding to the ONP stage) [86,87] and then

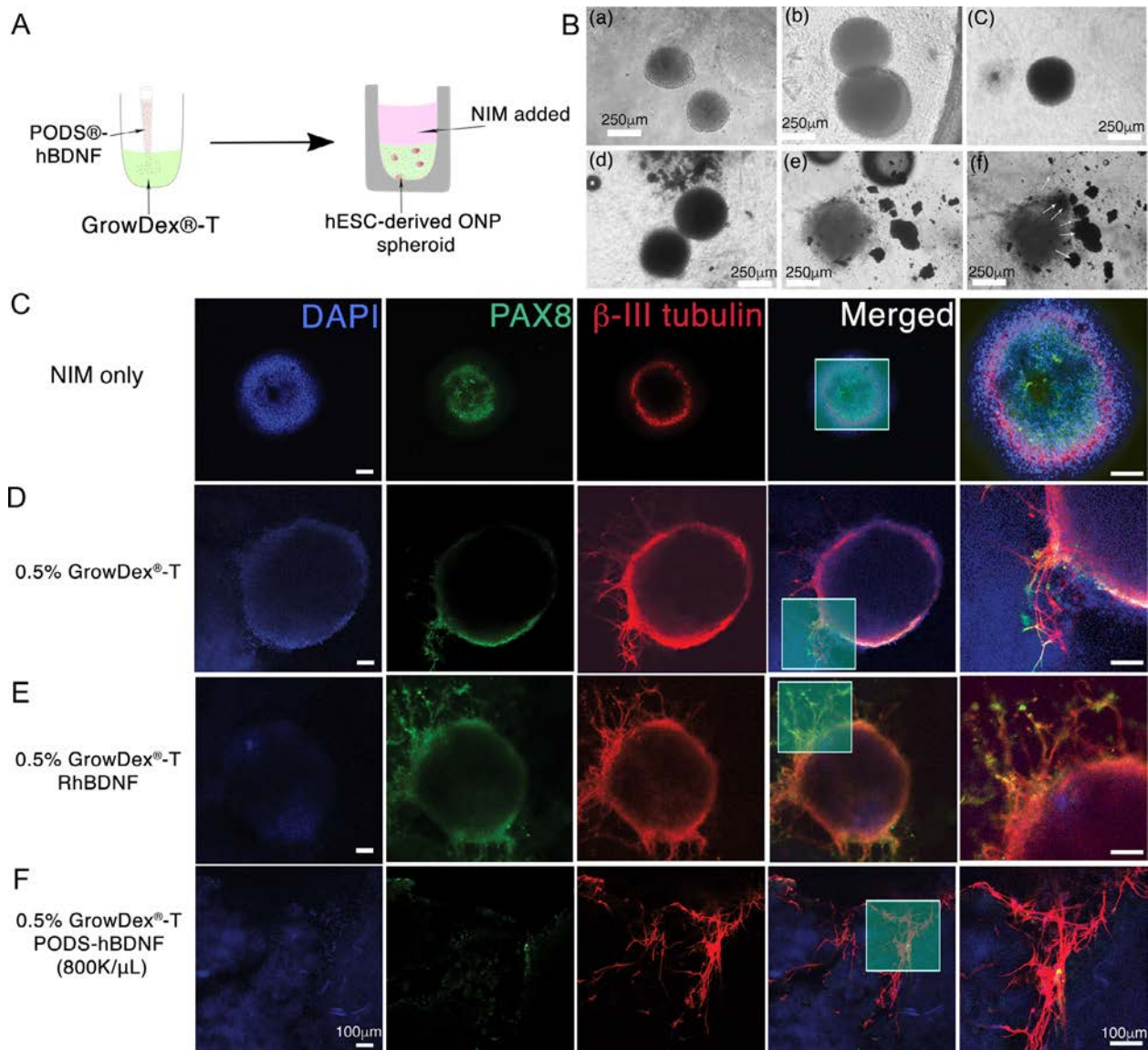


Fig. 6. Immunocytochemistry of hESC-derived ONP spheroids that were cultured with 0.5% GrowDex®-T *in vitro*. **(A):** Schematic diagram of PODS®-hBDNF and GrowDex®-T mixture with hESC-derived ONPs spheroids *in vitro*. **(B):** A phase-contrast photomicrograph of hESC-derived ONP spheroids cultured with GrowDex®-T (gray background) and/or PODS®-hBDNF (shown in black). Human ESC-derived ONP spheroids that were cultured with 1% GrowDex®-T for 24 h (a), 48 h (b), and 72 h (c), respectively. Human ESC-derived ONP spheroids that were cultured with 1% GrowDex®-T and 800,000/μL of PODS®-hBDNF for 24 h (d), 48 h (e), and 72 h (f), respectively. **(C):** Immunocytochemistry of hESC-derived ONP spheroids cultured in NIM for 7 days. **(D):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.5% GrowDex®-T for seven days. **(E):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.5% GrowDex®-T with 20 ng/mL of RhBDNF for seven days. **(F):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.5% GrowDex®-T with 800,000 of PODS®-hBDNF for 7 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to down-regulate afterward [86–88]. Fig. 7E demonstrates that PAX8-positive cells are significantly more common in NIM-only conditions ($97.7\% \pm 0.14$ for 0.5% GrowDex®-T and PODS®-hBDNF and $99.0\% \pm 0.44$ for 0.375% GrowDex®-T and PODS®-hBDNF; shown in green bars) compared to other conditions. This finding suggests that GrowDex®-T in conjunction with PODS®-hBDNF can facilitate otic neuronal differentiation. This finding is also apparent in the second rows of Fig. 6C–F and Fig. 7A–D, which show that the positivity of PAX 8 was most obvious under NIM-only conditions.

The quantification of neurite lengths in hESC-derived ONP spheroids demonstrates significantly longer neurites in the spheroids that were cultured in the combination of GrowDex®-T and PODS®-hBDNF (Fig. 7F). The number of neurite-bearing cells in the ONP spheroids was significantly smaller when they were cultured under NIM-only conditions (Fig. 7G). Finally, a similar

trend was observed in our neurite arborization assay as in the neurite growth assay (Fig. 7F) in that axonal branching was significantly greater in a culture condition with both GrowDex®-T and PODS®-hBDNF (Fig. 7H).

3.8. In vivo transplantation of human embryonic stem cell-derived otic neuronal progenitor spheroids into the inner ear

Fig. 8A shows the experimental paradigm of this part of the study. Prior to stem cell transplantation in the inner ear, both tone burst-evoked and click-evoked ABRs were used to assess the sensitivity of the inner ear to acoustic stimuli to confirm the efficacy of the DTR mouse model. Fig. 8B and 8C show exemplary data of typical tone burst-evoked ABR waveforms on a WT C57/BL6 mouse (Fig. 8B) and a deafened DTR mouse (Fig. 8C). A level- and

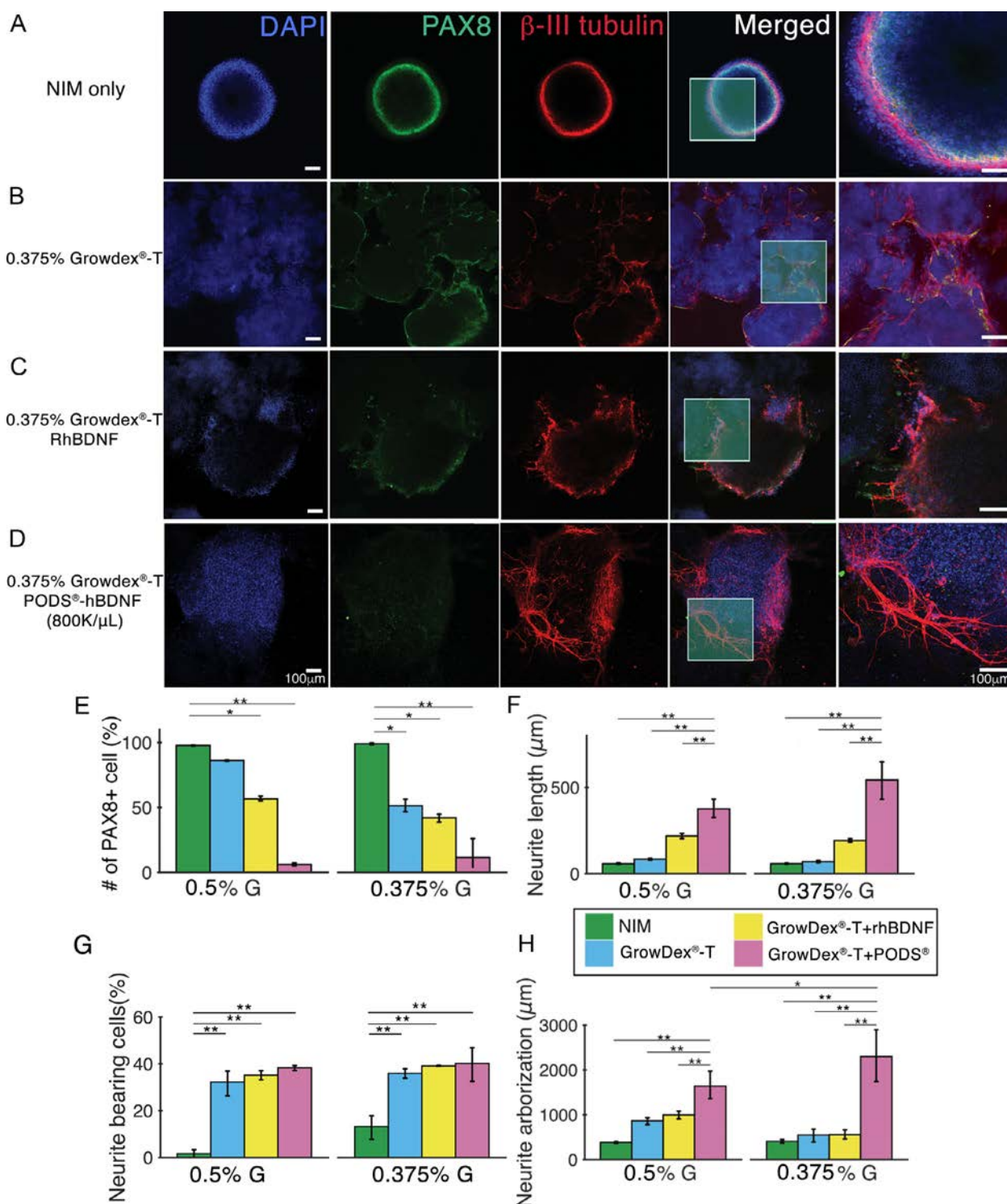


Fig. 7. Immunocytochemistry of hESC-derived ONP spheroids cultured with 0.375% GrowDex®-T *in vitro*. **(A):** Immunocytochemistry of hESCs-derived ONPs spheroids cultured in NIM for seven days. **(B):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.375% GrowDex®-T for seven days. **(C):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.375% GrowDex®-T with 20 ng/mL of RhBDNF for seven days. **(D):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.375% GrowDex®-T with 800,000 of PODS®-hBDNF for seven days. **(E):** Quantification of positive staining for PAX8 based on immunocytochemistry in hESC-derived ONP spheroids. **(F):** Quantification of neurite length arising from hESC-derived ONP spheroids ($n = 3$). **(G):** Quantification of neurite bearing analysis. **(H):** Quantification of neurite arborization analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey's *post-hoc* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

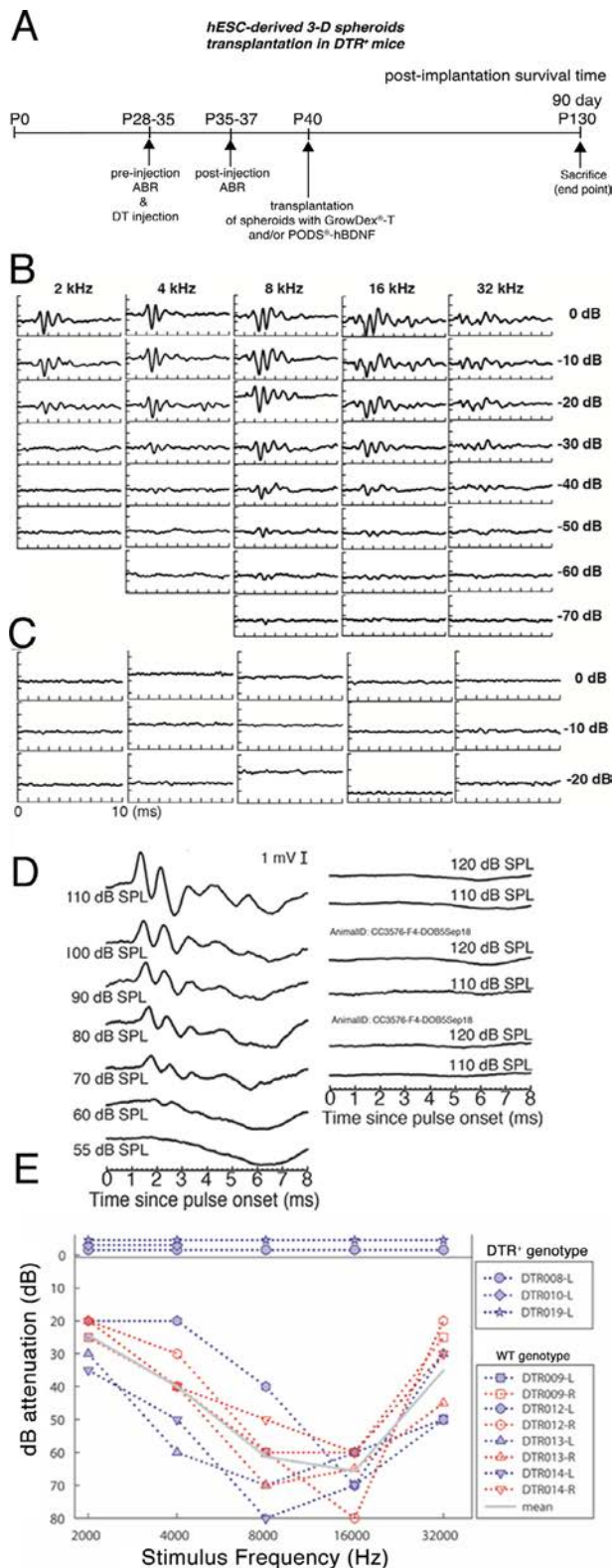


Fig. 8. *In vivo* hESC-derived ONP spheroid transplantation in the DTR mouse cochlea. **(A):** A schematic diagram of experimental design for *in vivo* hESC-derived ONP spheroid transplantation. **(B):** Tone-burst-evoked ABR assessment of a WT mouse. **(C):** Tone-burst-evoked ABR assessment of a DTR mouse. **(D):** Click-evoked ABR assessment of WT mouse (left column) and DTR mouse (right column). **(E):** ABR audiograms for 3 DTR and 8 WT mouse ears, with mean values for the latter shown with the gray line. Levels are expressed in terms of attenuation, with sound pressure levels at 0 dB ranging from 105 to 120 dB SPL. Thresholds were based upon visual detection of a response above the noise floor. No ABRs were detected for the three DTR mice (data plotted above the 0 dB line).

frequency-series of tone burst-evoked ABRs demonstrated that a DTR mouse had no detectable ABRs even at the lowest attenuation levels (i.e., 0 to -20 dB, equivalent to 100 to 120 dB SPL) (Fig. 8C). Fig. 8D shows exemplary data of click-evoked ABR waveforms for a WT mouse (left column) and a deafened DTR mouse (right column). The deafened DTR mouse demonstrated no detectable waves I or V even with the highest stimulation levels, indicating that it was adequately deafened for the study. Fig. 8E shows the plots of ABR threshold audiograms of three DTR mice injected with DT and of eight WT mice. For each DTR mouse, no ABRs were detected at any tested frequency. Fig. 9A shows immunohistochemistry of the cochleae of WT mice and DTR mice. Confocal fluorescence images of phalloidin (red) and myosin VIIa (green) in the whole-mounted auditory epithelium demonstrate intact HCs in both a WT and a DTR mouse without DT injection (control conditions are shown in Fig. 9A(a) and (b)). On the contrary, a P28 DT-treated mouse DTR with DT injection shows no signs of inner or outer HCs demonstrating ablation by DT throughout the cochlea (Fig. 9A(c) shows the basal turn and (d) the apical turn).

The hESC-derived ONP spheroids were then transplanted into the DTR mice cochleae.

After 90 days, hESC-derived ONP spheroids transplanted into the inner ear with 1% GrowDex®-T and PODS®-hBDNF were engrafted in the scala vestibuli (Fig. 9B(a)) and in the scala tympani (Fig. 9B(b-c)). As Fig. 9B shows, the transplanted cells still maintained aggregated forms in the inner ear. Fig. 9B(b) also demonstrates that hESC-derived spheroids lodged in the wall of the scala tympani and extended neurites toward the modiolus (white arrow). A dissociated form of stem cell transplantation can be found in Supplementary Fig. S5.

We then performed quantification of the engrafted hESC-ONP spheroids. The AHNA+ profiles indicate that the engrafted ONP spheroids tended to lodge more in the basal turn of the cochlea than the mid- or apical turns (Fig. 9C(a)). Also, hESC-derived ONPs with dissociated forms did not survive as efficiently as those with spheroid forms (Fig. 9C(b)). Engraftment was significantly better with GrowDex®-T and GrowDex®-T with PODS®-hBDNF (Fig. 9C(c)). Further quantitative analysis confirmed that GrowDex®-T with PODS®-hBDNF promoted the neurite extension of engrafted hESC-derived ONPs *in vivo* (Fig. 9C(d)). Finally, the overall survival rate of our transplanted spheroids with GrowDex®-T and PODS®-hBDNF after 90 days was approximately 0.1% (see the Supplementary Data for further details). We evaluated the presence of NFC hydrogel in the transplanted cochlea using Calcofluor White staining. Fig. 9D shows that the NFC hydrogel was present for 7 days (Fig. 9D(b)) and also 90 days (Fig. 9D(c)) after transplantation.

Finally, immunorejection was evaluated with H&E staining, as our transplant was xenografted (i.e., human stem cells to mice). Fig. 9E(a) shows a control cochlea without transplantation; that is, there was no areolar fibrous tissue or any lymphocyte infiltration noted. Fig. 9E(b) and (c) show an hESC-transplanted cochlea with GrowDex®-T and PODS®-hBDNF (on day 7 and 90, respectively). In Fig. 9E(b), there is mild areolar fibrous tissue infiltration in the scala vestibuli (red circle). Fig. 9E(c) shows lymphocyte aggregation and dilated blood vessels in the surrounding area of the transplanted cochlea but little overall cell response inside the membranous labyrinth.

Fig. 9F shows protein expression of MHC class I and MHC class II antigens assessed by flow cytometry in undifferentiated H9 hESCs (upper row) and hESC-derived ONP spheroids (lower row). Both undifferentiated H9 hESCs and hESC-derived ONP spheroids demonstrated minimum detection of MHC class I or MHC class II antigens. Positive-control of flow cytometry can be found in Supplementary Fig. S6.

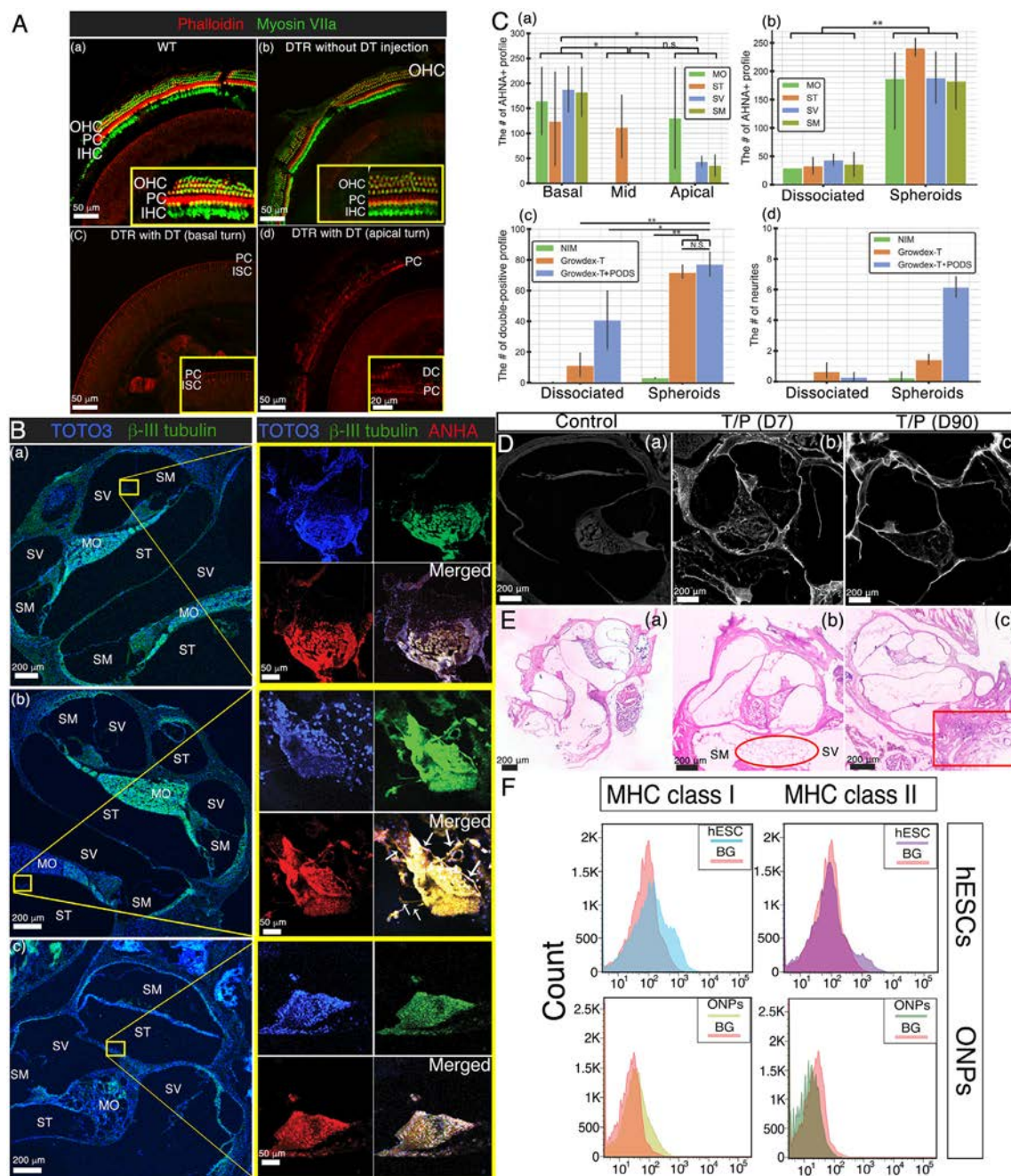


Fig. 9. (A): Immunohistochemistry of the DTR mouse cochlea. Confocal fluorescence Images of phalloidin (red) and myosin VIIa (green) in the whole mounted auditory epithelium. (a) A wild type cochlea (control) shows a single row of IHCs and three rows of OHCs in the basal turn. (b) A DTR mouse cochlea without DT injection also shows a single row of IHCs and three rows of OHCs in the basal turn as well. (c and d) A DTR mouse cochlea with DT injection at P25 demonstrates no IHCs or OHCs in the basal turn (c) and the apical turn (d). OHC: outer hair cells; IHC: inner hair cells; PC: pillar cells, DC: Deiters cells, and ISC: inner supporting cells. **B**): Immunohistochemistry of transplanted hESC-derived ONP spheroids in the DTR mouse cochlea. Left column: a corresponding low-power magnification microphotograph of the DTR mouse cochlea (10X). Each yellow circle indicates the anatomical location of a transplanted hESC-derived ONP spheroid. Right column: high-power magnification microphotographs of transplanted hESC-derived ONP spheroids stained with TOTO3 (nuclear counterstaining), β -III tubulin, and AHNA (40X). (a) Human ESC-derived ONP spheroids transplanted with 1% GrowDex®-T. (b) Human ESC-derived ONP spheroids transplanted with 1% GrowDex®-T and 800,000 of PODS®-hBDNF. Small white arrow: neurites. (c) Another hESC-derived ONP spheroid transplanted with 1% GrowDex®-T and 800,000 of PODS®-hBDNF. SM: scala media, SV: scala vestibuli, ST: scala tympani, MT: the middle turn of the cochlea, and MO: modiolus. **C**): (a) Quantification of the number of ANHA positive cells (profile) in three different turns of the DTR mouse cochlea. (b) Quantification of the number of the ANHA positive cells (profile) in dissociated ONPs transplantation vs. ONP spheroids transplantation in four anatomical subdivisions of the cochlea. (c) Quantification of the number of the triple positive cells (profile) in dissociated ONPs transplantation vs. ONP spheroid transplantation with three different substrates. (d): Quantification of the number of neurites in dissociated ONPs transplantation vs. ONP spheroid transplantation. NIM: neuronal induction media, G: GrowDex®-T, G + P: GrowDex®-T and PODS®-hBDNF. * $p < 0.05$, ** $p < 0.01$, N.S.: not significant by one-way ANOVA with Tukey's *post-hoc* test. Note that all of the images stained for TOTO3 iodide and AHNA have been pseudo-colored. **D**): Calcofluor White staining indicates the presence of NFC at both seven days and ninety days post-transplantation. **E**): Tissue response in H&E histology. (a) Control WT cochlea (no surgery). (b) DTR mouse cochlea transplanted with hESC-derived ONP spheroids with GrowDex®-T and PODS®-hBDNF. The DTR mouse was euthanized seven days after the transplant surgery (D7). (c) DTR mouse cochlea transplanted with hESC-derived ONP spheroids with GrowDex®-T and PODS®-hBDNF. The DTR mouse was euthanized 90 days after the transplant surgery (D90). **F**): Expression of MHC class I and MHC class II proteins assessed by flow cytometry in undifferentiated hESCs (H9) and hESC-derived ONP spheroids (ONPs). Orange lines represent background staining with the conjugated antibody (MHC I and MHC II) alone. Three independent experiments were performed and the results were averaged for each analysis. BG: background. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The number and integrity of surviving SGNs have been implicated in the hearing sensitivities of deafened patients reliant on CIs or future stem cell–replacement therapy [77,89,90]. A sustained ability to repopulate SGNs would enable improved clinical results [10,91–94]. Moreover, while HC regeneration is an important research area, successful inner-ear function will still depend on the effective transmission of electrical signals to the brainstem via SGNs. Therefore, new techniques to enhance the efficiency of stem cell–derived ONP transplantation to augment extant SGN populations are critical. Historically, the low overall survival of stem cell–derived ONPs transplanted into the inner ear can be attributed to its relatively inhospitable environment and its inadequate levels of trophic factors [26,30,63,95]. To overcome these issues, we created an artificial stem cell niche using hESC-derived ONP spheroids, NFC hydrogel, and a self-sustaining source of BDNF to promote the survival and neuronal differentiation of transplanted hESC-derived ONPs. We used hESCs-derived ONPs to generate spheroids, as their use is more clinically relevant to the core interests of the study than autologous murine stem cells. In addition, human and murine stem cells behave drastically different in culture. For instance, there are profound differences in cell cycle regulation, control of apoptosis, and cytokine expression between the two [96]. Given that the majority of ongoing clinical trials use human pluripotent stem cells, our work represents a significant step in advancing stem cell replacement therapy in the human inner ear.

Immunocytochemistry of our monolayer culture revealed that hESC-derived ONPs cultured with PODS®-hBDNF for seven days expressed adequate markers for human SGNs, a finding consistent with our previously reported data [56,63,70,87]. We also performed qRT-PCR, demonstrating that our hESC-derived ONP spheroids also had otic lineages, although not identical to human SGNs [56]. Notably, hESC-derived ONPs were mostly peripherin-positive, which is more consistent with type II human SGNs [97], although this is consistent with a previous result on mouse ESC-derived ONPs [98].

In addition, PODS®-hBDNF treatment on hESC-derived ONP spheroids showed that it promoted otic neuronal differentiation, as it significantly decreased nestin- and SOX2- positive cells; however, PAX8-positive cells did not significantly differ between RhBDNF treatment and PODS®-hBDNF treatment. Our immunocytochemistry data and qRT-PCR data on hESC-derived ONP spheroids, however, indicated significantly decreased PAX8-positive cells in PODS®-hBDNF and GrowDex®-T, suggesting that our artificial stem cell niche promoted further neuronal differentiation than a 2D-monolayer ONP culture with PODS®-hBDNF [74]. A qRT-PCR confirmed that hESC-derived ONP spheroids were guided toward otic glutamatergic neural lineages (Fig. 4H).

We performed a rheological analysis of NFC hydrogel for two reasons. First, the GrowDex®-T we used in this study was an anionic-charged NFC hydrogel, which is different than GrowDex® (neutrally-charged NFC hydrogel) [40]. The rheological characteristics of NFC hydrogel, including swelling properties, can be affected by the extent to which the hydrogel is ionized. Second, the original concentration of GrowDex®-T was 1%; however, microscopic visualization of the cells in 1% GrowDex®-T was extremely challenging, so we diluted it to 0.5% and 0.375% to determine the cells' rheological characteristics. The growing frequency sweep in the NFC hydrogels demonstrated that all the NFC hydrogel samples had approximately $\tan\delta$ (G''/G') ~ 0.08 – 0.13 (gel-like structures) and little frequency dependence, which is a characteristic of self-standing elastic gels. The influence of shear stress on the viscosity of the 1% and 0.5% NFC hydrogels indicated reversible gelation because fluid-like behavior was observed at high shear stress levels (>10 Pa for 0.5% and >20 Pa for 1% NFC hydrogel), whereas

gel-like behavior was observed at low shear stress levels. This finding is consistent with previous studies that used NFC hydrogel and other physiological hydrogels [40,42,99,100]. However, the 0.375% NFC hydrogel did not demonstrate this feature, presumably because of its high-water content.

A strain sweep of the NFC hydrogels indicated that both the 0.375% and 0.5% NFC hydrogels showed nearly linear deformation in the entire measured strain regions (1–15%) due to their higher water content. However, the 1% NFC hydrogel deviated from linearity above a 7% shear strain. This disproportionately higher shear modulus shows that the yield stress was not directly proportional to the concentration of the 1% hydrogel above a 7% shear strain. This finding can be attributed to the composition of 1% hydrogel containing more cellulose, which formed fiber-like aggregates at higher shear strains. This “shear thinning” behavior of the 1% hydrogel (which we used) made it an attractive ECM for our clinical application, as it allowed the encasing of hESCs in the gel in a low-shear force environment. NFC hydrogel also allowed smooth injections by syringe (high shear force) into the inner ear.

Our ELISA results showed a steady rate of release of RhBDNF from PODS® crystals at approximately 25–50 pg/mL/day, at which rate both *in vitro* and *in vivo* immunocytochemistry demonstrated more promotion of otic neuronal differentiation and enhanced axonal growth on transplanted ONPs than RhBDNF at 20 ng/mL/day. Our *in vitro* and *in vivo* results support the notion that, with the use of PODS®-hBDNF, BDNF concentrations of 25–50 pg/mL were sufficient for hESC-derived ONPs to differentiate into otic neuronal lineages.

Notably, the endogenous secretion of BDNF from hESC-derived ONPs was not detected by ELISA, either because hESC-derived ONPs did not actually secrete BDNF or the ONPs themselves consumed it. In other words, the release of 25–50 ng/mL/day of BDNF might have been greater because the ELISA measurement did not consider consumed BDNF (*i.e.*, by hESC-derived ONPs). While the exogenous provision of hBDNF with the polyhedrin delivery system promoted otic neuronal differentiation in our study, the degradation kinetics of PODS® crystals by any protease and long-term sequelae after more than 90 days is still undetermined. Further studies will be needed to investigate the endogenous provision of BDNF from hESC-derived ONPs.

Multicellular spheroids consist of viable cell rims and central necroses, which typically arise at spheroid diameters of 400–600 μm [101], a finding determined by fluorescence microscopy. Our hESC-derived ONP spheroids lacked fluorescence signals in their centers (Fig. 6–7), however. There are two possible explanations for this absence: (1) a limitation of the blood supply or removal of the waste product caused by hypoxia [101] or (2) a lack of fluorescent antibody penetration to the core. In the latter case, mixing a low number of PODS®-hBDNF crystals during ONP spheroid generation or reducing the total number of cells in individual spheroids may be methods to reduce the sizes of the central necroses. Further studies using hypoxia assays will be required to shed light on this matter.

Previous studies have shown that hESC-based stem cell therapy in the inner ear is hindered by poor engraftment [26,28,63]. The majority of transplanted, dissociated hESCs were either unable to attach to the lining of fluid-filled inner-ear scalae or, even if they attached, the lack of aggregation initiated apoptosis cascades, resulting in cell death [28,63]. After our *in vivo* hESC-derived ONP transplantation, ONPs were found in various scalae in aggregated formations presumably because of (1) the 3D nature of the transplanted ONP spheroids, (2) the NFC hydrogel facilitating their attachment to the lining of scalae, and (3) the continuous release of BDNF by a polyhedrin delivery system.

Our study demonstrated that hESC-derived ONP spheroids transplanted into artificial stem cell niches survived for 90 days at

a noteworthy survival rate of 0.1% without the use of immunosuppressant medication. While it is difficult to compare, most of the previous stem cell transplantation studies in the inner ear demonstrated a similar survival rate with much shorter survival duration (1–4 weeks) [26,28,30–32,102,103]. They also used immunosuppressants. The low immunogenicity of hESCs has at least three possible reasons. First, undifferentiated hESCs express low levels of MHC class I and class II molecules in both undifferentiated and differentiated states [104–106]. For this reason, we performed flow cytometry on our hESC-derived ONPs. Our results were consistent with those of previous studies (Fig. 9F). Second, hESCs lack costimulatory molecules, such as CD 80 and CD 86, which are essential for attacking transplanted hESCs in immunorejection responses [105]. Third, hESCs suppress dendritic cell-mediated T-cell proliferation [107]. Fig. 9E(c), for instance, shows a mild amount of lymphocyte aggregation in the surrounding tissue of the transplanted cochlea. Fourth, the inner ear is an “immune-privileged” organ due to its tight junction-based blood-labyrinthine barrier, similar to the eyes and the brain [108–110]. Finally, a mesh-size analysis of NFC hydrogels indicated that the average mesh size was ~35 nm [111]. The diameters of macrophages and lymphocytes (critical players in immunorejection) range from 20 to 30 μm in size, which is approximately 10^3 times greater than the mesh size, so the nanopore size of NFC hydrogels might have prevented immunorejection by blocking their entry. Further studies in the immune rejection will be required to investigate this issue before a clinical transition.

Human SGNs are bipolar neurons [69]; however, the hESC-derived ONP spheroids extended multipolar neurites *in vitro* (Fig. 7) and *in vivo* (Fig. 9). Our neurite arborization assay and neurite growth assay also demonstrates that the neurite branching was greater in a culture condition with GrowDex®-T and PODS®-BDNF (Fig. 7H). Ross and Burkel previously reported that unmyelinated SGNs tended to show multipolar morphology [112], and therefore, these multipolar neurites are more likely unmyelinated dendrites. Considering that our long-term aim is to generate a neuronal network connecting each of CI electrodes to extant SGNs, the larger dendritic tree that was shown in our study may, in fact, be pivotal in establishing the connection. A study in a feline retinal ganglion cell demonstrated that the shape and size of the receptive field are determined by the shape and size of underlying dendritic arborization [113]. They concluded that the larger the dendritic tree, the larger the visual area over which they can receive the input. Computational studies also support this finding [114,115]. If we translate this finding into the inner ear, the larger dendritic tree could receive more information from the CI electrode, which could potentially result in a lower threshold for electrical stimulation, a lower stimulation-current level requirement, and potentially higher spatial resolution (not so much for temporal resolution) of a biohybrid CI. Therefore, the higher arborization of neurites (unmyelinated dendrites) shown in this study may prove beneficial to our aim even though their shape is not bipolar. Further studies such as electrically-evoked ABR would give us more insight on this issue.

One of the limitations of this study was our immunohistochemistry. Due to the technical nature of AHNA, the antigen retrieval process was necessary, during which time our specimens were incubated under 120°C for 30 min. This treatment often distorted the thin-sliced specimens (Fig. 9). For future experiments, we recently developed and established lenti-viral transfected fluorescent-positive hESC-derived ONPs to avoid antigen retrieval, improving image quality. Another limitation is that our ESC-derived ONP spheroids may have possessed molecular profiles similar, but not identical, to human SGNs. All of the markers we used in this study are not otic lineage specific. In addition, we have not performed any immunohistochemistry or qRT-PCR to distinguish our ONP spheroids from vestibular lineages. Once again, further functional studies such as electrically-evoked ABR would give us more in-

sight into determining how much similarity in molecular profile is required for transplanted hESC-derived neurons to support a newly generated neuronal network within the inner ear.

On a technical note, hESC-derived ONP spheroid injections without the use of NFC hydrogel were not possible due to the constant backflow of perilymph from the scala tympani in the mice. The use of 0.375% or 0.5% of GrowDex®-T for injections did not allow any hESC-derived ONP spheroids to advance further in the scala tympani, presumably due to low viscosity and the stiffness of the gel. The use of 1% GrowDex®-T was chosen because its high viscosity and stiffness make injections feasible, allowed the hESC-derived ONPs to advance further into the scala tympani toward the mid-turn to secure placement, and displaced some transplanted ONPs in the scala media and the scala vestibuli. A future *in vivo* study may need to adjust the stiffness and viscosity of NFC hydrogel to place spheroids in the scala tympani and keep them localized despite the constant perilymphatic outflow.

5. Conclusions

Taken together, our data demonstrated that transplanted hESC-derived ONP spheroids survived, neuronally differentiated into otic neuronal lineages, and extended neurites toward the bony wall of the cochlea. Our data *in vitro* and *in vivo* presented here provide sufficient evidence that we have established a robust, reproducible protocol for *in vivo* transplantation of hESC-derived ONPs to the inner ear that may pave the way toward a next-generation “biohybrid” CI that combines the proven CI approach with hESC-replacement therapy [20]. Using our protocol to create an artificial stem cell niche in the inner ear using a 3D spheroid formation, NFC hydrogel for ECM, and a polyhedrin delivery system for the continuous provision of BDNF, it is once again possible to work toward integrating transplanted hESC-derived ONPs further into the inner ear as well as toward achieving functional auditory neurons generated from hESCs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.actbio.2020.03.007](https://doi.org/10.1016/j.actbio.2020.03.007).

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A COMSOL Multiphysics® Finite Element Model of the Diffusion Profile of Brain Derived Neurotrophic Factor: Biological Validation through a Microfluidics Device

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INTRODUCTION:

Sensorineural hearing loss can be treated in many cases by regenerating the synaptic connections between the extant Auditory Neurons (ANs) and transplanted human stem cell-derived ANs. One growth factor that plays an essential role in this paradigm is Brain-Derived Neurotrophic Factor (BDNF), integral to both cell survival and directed growth of AN peripheral processes (neurites). Using COMSOL Multiphysics® simulation software, we have developed a finite element model to analyze the diffusion profile of varying concentrations of BDNF released from the Polyhedrin Delivery System (PODS™) (Cell Guidance System, Cambridge, U.K) within a Xona Microfluidics device. We will then compare it to empirical experiments using immunohistochemistry as a measurement tool to assess for cell survival, migration and directed neurite growth.

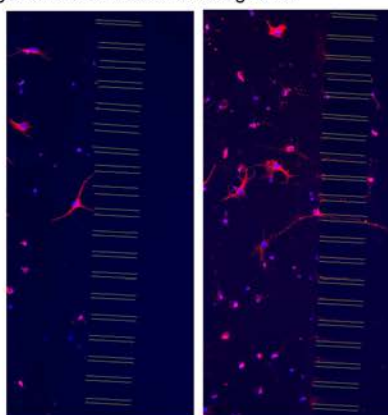
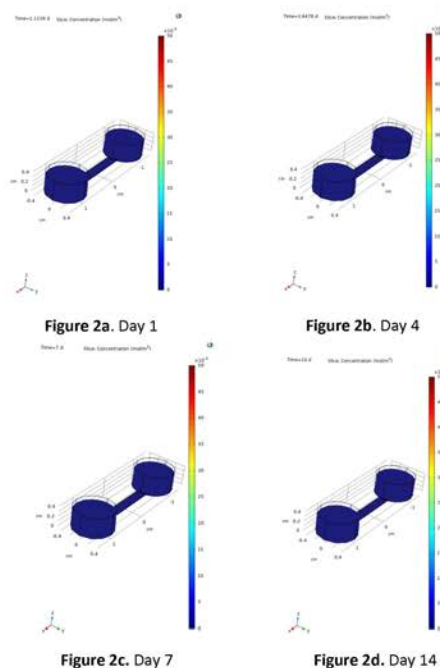


Figure 1: XC150 Immunocytochemistry (after 7 days culture). Stained with DAPI and β -III Tubulin. (A) Image of differentiated auditory neurons of positive control (cells with exogenous human recombinant BDNF). Image shows minimal cell migration and neurite extension through microchannels. (B) Image of differentiated auditory neurons of experimental condition (cells with 3.5 million PODS). Image shows marked cell migration and neurite extension along the device.

COMPUTATIONAL METHODS:

The geometry for the mesh was created using the native geometry builder of the COMSOL Multiphysics® software. We characterized the reaction that describes the breakdown of the polyhedrin-BDNF peptide link as a heterogeneous chemical reaction occurring at a predefined surface, in which the reactants are in distinct phases (e.g. solid phase and liquid phase), for which the Chemical Reaction Engineering module is directly applicable. The BDNF PODS (reactant) were modeled as being adsorbed to the predefined surface boundary, while the cell-secreted proteases (catalyst) were modeled as a bulk species. The second reaction is the subsequent natural degradation of the product (free BDNF). Rate constants for either reaction were approximated by regression analysis performed on quantitative data attained by measuring the sustained release profile of BDNF released by PODS, and as well as the degradation of recombinant BDNF over time. The simulation was computed using the chemistry, transport of diluted species, and surface reaction interphases in a time-dependent study.

RESULTS:



CONCLUSIONS:

Our computational models can help predict the optimal parameters needed to achieve AN neurite growth *in vivo*. Standard estimates state that 10 ng/mL of BDNF is needed for sustained neurite growth, so the parameters in our simulation experiment would work with that. Future directions of the work involve conducting similar simulations using the geometry of the inner ear with validation by empirical *in vivo* experiments in live mice.



Fig 3: (a): Conventional cochlear implant (Cochlear, LTD). **(b):** Mouse cochlear tissue sample that was imaged using 22 keV X-rays at Argonne National Laboratory.

ACKNOWLEDGEMENTS:

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2. Thorne, M., Salt, A., DeMott, J., Henson, M., Henson, O., Gewalt, S., Cochlear Fluid Space Dimensions for Six Species. Derived From Reconstructions of Three-Dimensional Magnetic Resonance Images, *The Laryngoscope*, 109, 1661-1668 (1999).

Stem Cell Regeneration of Human Spiral Ganglion Neurons toward Hearing Restoration



PI: Akihiro J. Matsuoka, M.D., D.M.Sc., Ph.D.

Co-Investigator: Eri Hashino, Ph.D.

Award: \$1,420,283

Org: Northwestern University Feinberg School of Medicine (NU)

Indiana University School of Medicine (IUSM: consortium)

Study Aims

- Optimize the delivery of human stem-cell-derived auditory neurons to the cochlea.
- Assess how well three bio-engineered support strategies improve survival and growth of those neurons using an *in vitro* (culture dish) test bed.
- Refine the results by assessing the strategies in an animal model. Use a genetically engineered mouse (providing a refined deaf-cochlea model) to transplant stem-cell-derived neurons and the support strategies into the deaf cochlea.
- Key outcome measures include cell survival, neurite growth, auditory neuronal differentiation, and synapse formation.

Approach

- Two cell-delivery methods (based on controlling pressure or flow) will be tested.
- Use spheroids and organoids, both containing stem-cell-derived neurons, to assess the hypothesized positive effects of grouping cells into 3-D aggregates.
- Use our proven method for converting human embryonic stem cells to neurons.
- Also assess the supportive effects of an extracellular support matrix and a neurotrophin, i.e., a protein known to support neuron health.
- The *in vitro* model provides optimization of neurotrophin concentration.
- The *in vivo* model provides study of cell survival, neuronal differentiation, and synaptic plasticity across time.

DESIGN

Hypothesis: Biotechnologies promote growth and neural connections in ears transplanted with human stem-cell derived neurons.

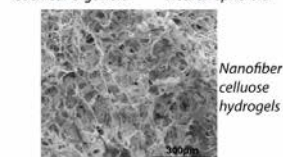
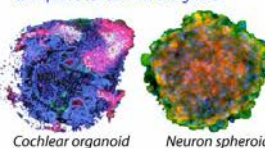
3D cell aggregates

Supportive extracellular matrix

Controlled neurotrophin delivery via crystal attachment

BIOTECHNOLOGIES

Three biotechnologies chosen to improve cell survival and growth



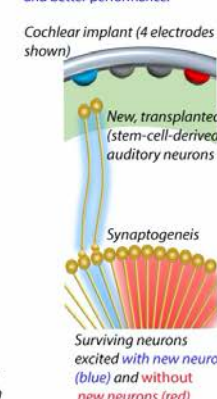
EVALUATION

Are cell survival and synaptic connections better? Use *in vitro* and *in vivo* models



CLINICAL GOAL

The biobrid cochlear implant combines new neurons and CI electrodes for more precise stimulation and better performance.



- Accomplishments:** (1) Published protocol to direct stem cells to auditory neuron fate
(2) Evaluated extracellular matrix to support neuron survival (manuscript submitted)
(3) Successful cochlear implantation of stem-cell-derived neurons and spheroids

Timeline and Cost

Major Activities		2018	2019	2020	2021
Aim 1 & 2 Generation of inner-ear organoids (IUSM) and neuronal spheroids (NU)					
Aim 1 Optimize cell transplantation methods					
Aim 2 Generation of BRN3A 2A-ntdTomato reporter hESC line (IUSM)					
Aim 2 Assess <i>in vitro</i> niche effectiveness					
Aim 2 Assess <i>in vivo</i> niche effectiveness					
Data dissemination	Meetings		TERMIS ISSCR COMSOL	ISSCR	ARO ISSCR TERMIS
	Publication prep/submit				
Estimated Budget (\$1.4M)		0.10 M	0.40M	0.40M	0.50M

Updated: Oct 31, 2020

Goals / Milestones

CY18 Goal – Optimize cell transplantation and begin *in vitro* testing

- ✓ Perform LIVE/DEAD assay to optimize cell transfer techniques
- ✓ Begin *in vitro* evaluation of support strategies (niches) for derived neurons

CY19 Goals – *In vitro* & *in vivo* evaluation of supportive niches

- ✓ Continue *in vitro* evaluations begun in CY18
- ✓ Begin *in vivo* studies of transplanted neuron survival, neurite growth, and synaptic connectivity using the genetically engineered “DTR” animal model

CY20 Goal – Continue evaluations using DTR mouse model

- Optimize BDNF (neurotrophin) concentrations for use with *in vivo* model.
- ✓ *in vivo* assessment of transplanted hPSC-aggregates

CY21 Goal – Completion of work: Results characterization

- Final assessments: neuron survival, neurite growth, auditory neuronal differentiation, and synaptogenesis
- Final assessment: effects of post-implantation time on neuron survival

Comments/Challenges/Issues/Concerns

- The COVID 19 pandemic is affecting our progress since March 2020.

Budget Expenditure

Projected Expenditure: \$1,420,283

Actual Expenditure: \$721,890.83 (10/31/2020)

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Appendix to the Specification	nu-1703-appendixd.pdf	3980470	no	62
			b74d30bfab6ae445f524dada48c105d271efc58f		
Warnings:					
Information:					
2	Specification	nu-1703-specification.pdf	132152	no	6
			781d4d2c929caf0422c8e8ca04228f848892e47c		
Warnings:					
Information:					
3	Application Data Sheet	nu-1703-ADS.pdf	108242	no	6
			1f01030d75fc0d035105609f690a7d6b3986be19		
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
4	Fee Worksheet (SB06)	fee-info.pdf	30327	no	2
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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

**AN ENGINEERED THREE-DIMENSIONAL STEM CELL NICHE IN THE INNER EAR
BY APPLYING A NANOFIBRILLAR CELLULOSE HYDROGEL WITH A
SUSTAINED-RELEASE NEUROTROPHIC FACTOR DELIVERY SYSTEM**

BACKGROUND AND SUMMARY

[0001] The field of the invention relates to compositions and methods for preparing and using stem cell niches. In particular, the field of the invention relates to compositions and methods for preparing and using engineered three-dimensional stem cell niches for insertion in the inner ear.

EXAMPLES

[0002] The following Examples are illustrative and should not be interpreted to limit the scope of the claimed subject matter.

[0003] Example 1

[0004] Please see enclosed manuscript by Chang *et al.* entitled "An Engineered Three-Dimensional Stem Cell Niche in the Inner Ear by Applying a Nanofibrillar Cellulose Hydrogel with a Sustained-Release Neurotrophic Factor Delivery System," the content of which is incorporated herein by reference and forms part of this application.

[0005] It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and

expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been illustrated by specific embodiments and optional features, modification and/or variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00109] Citations to a number of patent and non-patent references may be made herein. The cited references are incorporated by reference herein in their entireties. In the event that there is an inconsistency between a definition of a term in the specification as compared to a definition of the term in a cited reference, the term should be interpreted based on the definition in the specification.

CLAIMS

We claim:

1. An artificial stem cell niche (microenvironment) capable of being implanted into the inner ear comprising:
 - a) 3-D hPSC-derived otic neuronal progenitor (ONP) spheroid;
 - b) nanofibrillar cellulose hydrogel; and
 - c) a sustained release delivery system comprising brain-derivative neurotrophic factor (BDNF).
2. The artificial stem cell niche of claim 1, wherein the ONP spheroids are embedded in the nanofibrillar cellulose (NFC) hydrogel.
3. The artificial stem cell niche of any one of the preceding claims, wherein the sustained release delivery system is a polyhedron protein delivery system.
4. The artificial stem cell niche of any one of the preceding claims, wherein the sustained release delivery system release about 20 pg/ml to about 100 pg/ml per day, preferably about 25 pg/ml to about 50 pg/ml a day.
5. The artificial stem cell niche of any one of the preceding claims, wherein the NFC hydrogel is about 0.5% to about 1.5% NFC hydrogel, preferably 1% NFC hydrogel.
6. The artificial stem cell niche of any one of the preceding claims, wherein the ONP spheroid has a density of about 30,000 to about 70,000 cells/spheroid.
7. The artificial stem cell niche of any one of the preceding claims, wherein the ONP spheroids have a diameter about 400-600 μm .
8. The artificial stem cell niche of any one of the preceding claims, wherein the ONPs express PAX8.

9. The artificial stem cell niche of any one of the preceding claims, wherein the ONPs express one or more additional markers selected from the group consisting of GATA3, Neurogenin-1 (NeuroG1), SOX2, nestin, vesicular glutamate transporter-2 (VGLUT2), β -III tubulin, and peripherin, and lack expression of the markers E-cadherin (ECAD), PAX6 and SOX10.

10. The artificial stem cell niche of any one of the preceding claims, wherein the ONPs were differentiated from human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC).

11. The artificial stem cell niche of claim 10, wherein the iPSCs are derived from a subject which is hard of hearing.

12. A method of making an artificial cell niche for implantation in the cochleae of a subject, the method comprising embedding hPSC-derived otic neuronal progenitor (ONP) spheroids into a nanofibrillar cellulose (NFC) hydrogel.

13. The method of claim 12, wherein the NFC hydrogel is about 0.5% to about 1.5% NFC hydrogel, preferably 1% NFC hydrogel.

14. The method of claim 11 or 12, wherein the hPSC are derived from the subject in need of a cochlear implant.

15. An *in vivo* method to create an artificial stem cell niche in the inner ear of a subject comprising:

implanting the artificial niche of claim 1 into the cochleae of a subject.

16. The method of claim 15, wherein the ONPs are derived from the subject in which the artificial niche is being implanted.

17. The method of claim 15 or 16, wherein the method further comprises:

implanting an electrode array capable of stimulating the nerves within the cochlea.

18. The *in vivo* method of claim 12, wherein the artificial stem cell niche is implanted on the epithelial layer of the scala tympani of the cochleae of the subject.

19. A biohybrid cochlear implant comprising:
the artificial cell niche of claim 1 capable of being implanted into the cochlear of a subject, and
a cochlear implant comprising an external portion and an implantable portion,
(a) the external portion comprising
(i) a microphone,
(ii) a speech processor,
(iii) a transmitter, and
(b) the implantable portion comprising a receiver and an implantable electrode array.

ABSTRACT OF THE DISCLOSURE

Disclosed are compositions and methods for preparing and using stem cell niches. Particularly disclosed are compositions and methods for preparing and using engineered three-dimensional stem cell niches for insertion in the inner ear.

Manuscript Number:

Title: An Engineered Three-Dimensional Stem Cell Niche in the Inner Ear
by Applying a Nanofibrillar Cellulose Hydrogel with a Sustained-Release
Neurotrophic Factor delivery System

Article Type: Full length article

Section/Category: Opt in to First Look

Keywords: Keywords: human embryonic stem cells; stem cell niche; spiral
ganglion neurons; the inner ear; stem cell-replacement therapy; hydrogel;
human pluripotent stem cells; brain-derived neurotrophic factor

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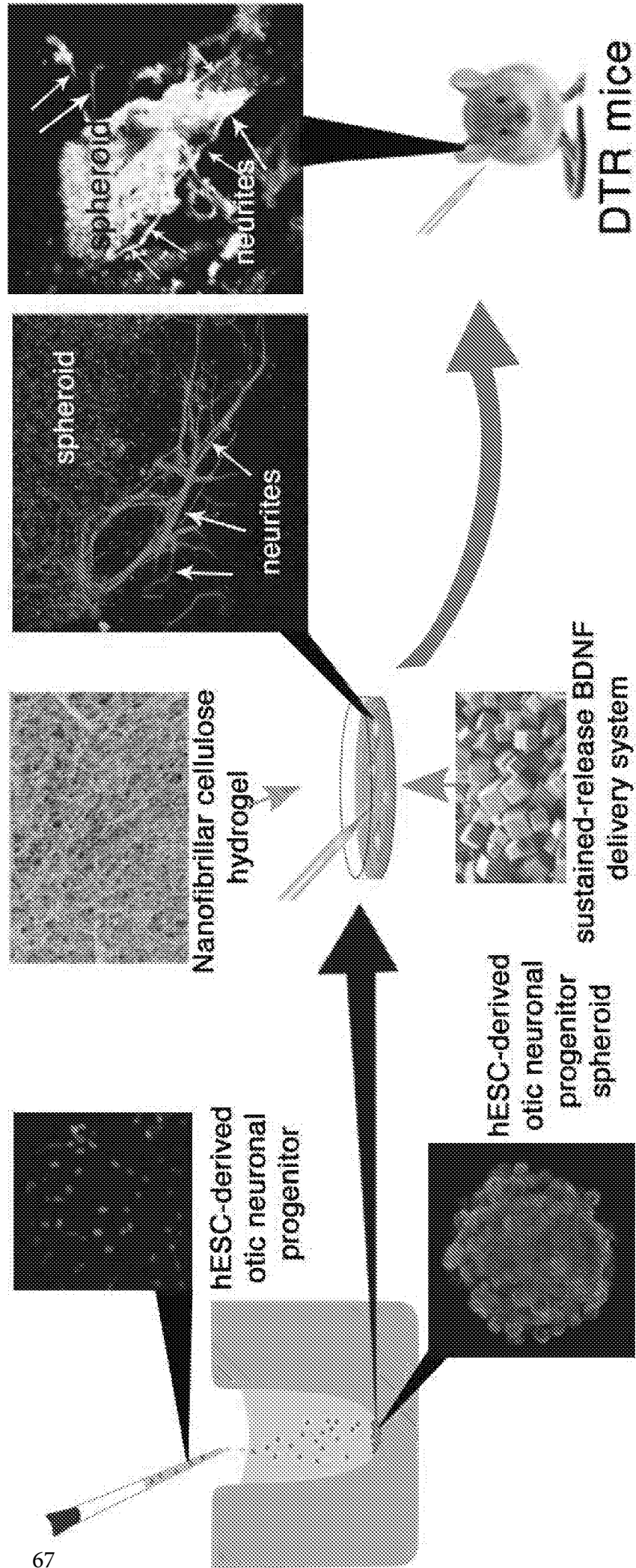
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Abstract: Although the application of human embryonic stem cells (hESCs)
in stem cell-replacement therapy remains promising, its potential is
hindered by low cell survival rate in post-transplantation within the
inner ear. Here, we aim to enhance the in vitro and in vivo survival rate
and neuronal differentiation of otic neuronal progenitors (ONPs) by
generating an artificial stem cell niche consisting of three-dimensional
(3D) hESC-derived ONP spheroids with a nanofibrillar cellulose hydrogel
and a sustained-release brain-derivative neurotrophic factor delivery
system. Our results demonstrated that the transplanted hESC-derived ONP
spheroids survived and neuronally differentiated into an otic neuronal
lineage in vitro and in vivo, and extended neurites toward the bony wall
of the cochlea in vivo. Our data in vitro and in vivo presented here
provide sufficient evidence that we have established a robust,
reproducible protocol for in vivo transplantation of hESC-derived ONPs to
the inner ear. Using our protocol to create an artificial stem cell niche
in the inner ear, it is now possible to work on integrating transplanted
hESC-derived ONPs further and also to work toward achieving functional
auditory neurons generated from hESCs. Our findings suggest that the
provision of an artificial stem cell niche is a fruitful future approach
to stem cell-replacement therapy for inner ear regeneration.

Statement of Significance

Inner ear regeneration utilizing human embryonic stem cell-derived otic neuronal progenitors (hESC-derived ONPs) has remarkable potential for treating sensorineural hearing loss. However, the local environment of the inner ear requires a suitable stem cell niche to allow hESC-derived ONP engraftment as well as neuronal differentiation. To overcome this obstacle, we utilized three-dimensional spheroid formation (direct contact), nanofibrillar cellulose hydrogel (extracellular matrix), and a neurotrophic factor delivery system to artificially create a stem cell niche *in vitro* and *in vivo*. Our *in vitro* and *in vivo* data presented here provide sufficient evidence that we have established a robust, reproducible protocol for *in vivo* transplantation of hESC-derived ONPs to the inner ear.



An Engineered Three-Dimensional Stem Cell Niche in the Inner Ear by Applying a Nanofibrillar Cellulose Hydrogel with a Sustained-Release Neurotrophic Factor Delivery System

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Abstract

Although the application of human embryonic stem cells (hESCs) in stem cell-replacement therapy remains promising, its potential is hindered by low cell survival rate in post-transplantation within the inner ear. Here, we aim to enhance the *in vitro* and *in vivo* survival rate and neuronal differentiation of otic neuronal progenitors (ONPs) by generating an artificial stem cell niche consisting of three-dimensional (3D) hESC-derived ONP spheroids with a nanofibrillar cellulose hydrogel and a sustained-release brain-derivative neurotrophic factor delivery system. Our results demonstrated that the transplanted hESC-derived ONP spheroids survived and neuronally differentiated into an otic neuronal lineage *in vitro* and *in vivo*, and extended neurites toward the bony wall of the cochlea *in vivo*. Our data *in vitro* and *in vivo* presented here provide sufficient evidence that we have established a robust, reproducible protocol for *in vivo* transplantation of hESC-derived ONPs to the inner ear. Using our protocol to create an artificial stem cell niche in the inner ear, it is now possible to work on integrating transplanted hESC-derived ONPs further and also to work toward achieving functional auditory neurons generated from hESCs. Our findings suggest that the provision of an artificial stem cell niche is a fruitful future approach to stem cell-replacement therapy for inner ear regeneration.

Keywords: Human embryonic stem cells; stem cell niche; spiral ganglion neurons; the inner ear; stem cell -replacement therapy; hydrogel; human pluripotent stem cells

1. Introduction

Between 25 and 48 million United States (US) residents are afflicted with sensorineural hearing loss (SNHL), which is usually an irreversible condition [1,2]. SNHL typically results from hair cell (HC) loss, initiating a cascade of trans-synaptic degeneration and the secondary loss of first-order neurons—spiral ganglion neurons (SGNs), the obligatory links to the brain. The degeneration of SGNs follows a distal-to-central progression, with peripheral processes degenerating first [3–5]. This pattern has negative implications for successful hearing restoration through the cochlear implant (CI), the only currently available treatment of SNHL [6,7].

Stem cell–replacement therapy with human embryonic stem cells (hESCs) has been proposed for restoring damaged HCs and SGNs [8,9]. However, HC replacement is technically challenging as precise placement in the difficult-to-access scala media is necessary—the diameter is only 600–800 μm in humans [10]. Furthermore, HC replacement therapy would still require the restoration of SGN peripheral processes and synaptogenesis. Unfortunately, the most promising report to date of stem cell-based therapy did not show broad or cochlear-specific restoration of HC-SGN synapses, nor has this report been replicated [11,12]. Part of the challenge arises from the inhospitable nature of the inner ear environment toward transplanted stem cells.

Stem cells normally reside in microenvironments (“niches”) that interact with surrounding cells to regulate and promote proliferation, differentiation, and survival [13,14]. While many questions remain as to which stem cell niche components are fundamental for hESCs to adequately and efficiently differentiate to SGNs, in general, the key players of a stem

cell niche include cellular components, an extracellular matrix (ECM), and secreted factors [15,16]. In the case of the inner ear, which consists of three primarily fluid-filled chambers—the scala tympani, scala media, and scala vestibuli—all three constituents of a stem cell niche are absent, creating an inhospitable environment for transplanted hESC-derived ONPs [17]. Indeed, previous efforts have been made to transplant hESC-derived ONPs or mouse mesenchymal stem cell-derived ONPs into these chambers or even directly into the modiolus where SGNs are housed [18–20]. The survival rate of the transplanted cells was, however, marginally low—on the order of 0.1–1.0 % [18,20]. Therefore, the successful use of stem cell therapy in the inner ear may require the creation of a supportive artificial stem cell niche within the inner ear, although the precise constituents of the normal microenvironment in the inner ear are still unknown.

When transplanted cells are injected into the scala tympani in a dissociated form, the cells first float in the perilymph within the scala tympani and then individually adhere to a layer of squamous epithelium that covers the scala tympani circumferentially [21]. The lack of direct cell contact with other transplanted stem cells, as in this scenario, can result in insufficient *bona fide* cell-cell adhesion molecule secretion and receptor interaction with membrane-bound ligands, leading to poor engraftment and inefficient neuronal differentiation [16]. To circumvent the lack of direct contact in transplanted hESC-derived ONPs in the inner ear, we have generated hESC-derived ONP spheroids [16,22]. A spheroid is defined here as a self-organizing multicellular structure of stem cells consisting of organ-specific progenitors that allow transplanted cells to maintain direct cell contact among each other, thereby maintaining

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4 the secretion of cell-cell adhesion molecules and receptors [23]. We hypothesize that this
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7 spheroid structure will promote the survival of cells and facilitate otic neuronal differentiation.
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10 Implantation of a hydrogel ECM can also provide a supportive stem cell niche by
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12 integrating a mechanical scaffold with the scala's squamous epithelium [24–26]. ECMs can hold
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14 transplanted stem cells in place and localize cell-to-cell signaling. Their integration with
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16 spheroids can reduce undesirable agglomeration and insulate cells from injection shearing
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18 forces that may lead to apoptosis [27]. Mammalian-derived collagens are commonly used;
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20 however, they are vulnerable to enzymatic degradation *in vivo*, making it challenging to create
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22 sustainable niches [28]. Fortunately, non-mammalian-derived hydrogels, such as nanofibrillar
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24 cellulose (NFC) hydrogel, can mimic native soft tissue ECMs in fiber size and mechanical
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26 properties [29–31]. An NFC hydrogel is also injectable and thus capable of delivering cells (i.e.,
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28 hESC-derived spheroids) to the desired target. Cellulose is biocompatible showing moderate
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30 foreign body responses, if any, and is safe for stem cell applications, with no known toxicity to
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32 hESCs [31–33]. Therefore, we hypothesize that utilizing an NFC hydrogel ECM will provide a
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34 supportive stem cell niche in the inner ear for hESC-derived ONP spheroids.
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43 Conventional growth factors, while necessary and effective as culture media
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45 supplements, suffer from fragility and thermo-instability under normal physiological conditions
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47 both *in vitro* and *in vivo*, resulting in short half-lives typically ranging from minutes to hours
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49 [34]. So far, this drawback has been addressed by large overprovisions of the requisite growth
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51 factor(s) and, in *in vitro* cultures, by frequent media changes to maintain a reservoir of bioactive
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53 protein in the culture medium. The polyhedrin delivery system (PODS®) is a crystalline growth
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55 factor formulation that has been developed to overcome the limitations of standard soluble
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4 growth factors. The PODS® technology has adapted viral machinery to encase chosen cargo into
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6 polyhedrin protein cases, creating PODS® growth factor proteins [35–37]. The resulting growth
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8 factor co-crystals have a slow degradation profile under physiological conditions and therefore
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10 allow the sustained release of the embedded bioactive growth factor protein. We focus on
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12 brain-derived neurotrophic factor (BDNF), which promotes survival and neuronal differentiation
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14 of SGNs *in vivo* and *in vitro* [38–40]. Degradation of the PODS® crystals is facilitated by cell- and
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16 protease-mediated processes, allowing the release of physiologically relevant levels of cargo
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18 protein (i.e., BDNF) over several weeks. We hypothesize that PODS®-human BDNF (hBDNF) will
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20 stably provide BDNF to hESC-derived ONPs, facilitate otic neuronal differentiation, and improve
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22 engraftment of transplanted cells.
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30 The purpose of this study is to create an artificial stem cell niche using hESC-derived
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32 ONP spheroids, NFC hydrogel, and a self-sustaining source of BDNF that promotes the survival
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34 and neuronal differentiation of transplanted hESC-derived ONPs. In this study, we first
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36 examined the effects of NFC hydrogel and the polyhedrin delivery system on hESC-derived ONP
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38 spheroids *in vitro*, followed by *in vivo* analysis using diphtheria toxin receptor-positive mice
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40 (DTR mice), a novel transgenic mouse model [41–43]. In the DTR mouse model, the gene for
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42 human diphtheria toxin receptor (hDTR) was inserted under the regulation of the promoter for
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44 *Pou4f3*, an HC-specific transcription factor [41]. In the DTR mouse model, a single injection of
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46 diphtheria toxin (DT) kills HCs, thus providing a suitable model for studying *in vivo* survival and
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48 neuronal differentiation of transplanted hESC-derived ONPs in a damaged cochlea. Here, we
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50 characterized the survival and neuronal differentiation of hESC-derived ONPs in an artificial
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52 stem cell niche. For this purpose, we used immunocytochemistry and immunohistochemistry
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4 along with the viscoelastic and rheological analysis of NFC hydrogel. Our findings suggest that
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6 this combination of factors may enable a future fruitful approach to stem cell replacement
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8 therapy for inner ear regeneration.
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11 **2. Materials and Methods**

12 *2.1. Differentiation of human embryonic stem cells into otic neuronal progenitors*

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14 Human ESC lines H1, H7, and H9 were used in this study (WiCell, Madison, WI, USA).
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16 ONPs were generated based on our previously established protocol [44]. See also the
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18 Supplemental Data in detail. A stepwise series of ligands and growth factors were added to a
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20 neuronal induction medium (NIM) to promote hESC differentiation towards a late-stage ONP
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22 lineage (Fig.1).
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30 *2. 2. Three-dimensional spheroid culture with nanofibrillar hydrogel*

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32 Human ESC-derived late-stage ONPs were passaged to a 96-well Clear Round Bottom
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34 Ultra-Low Attachment Microplate® (#7007; Corning Life Science, Tewksbury, MA, USA) at
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36 50,000 cells per well and centrifuged at 900 rpm for 20 minutes followed by incubation at 37°C
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38 for two days. Late-stage ONP spheroids were also generated by seeding 1 million cells into an
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40 EZSPHERE® culture plate (#2641701; Nacalai USA, CA, USA) at 37°C for two days. A 96-well Flat-
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42 Bottom Clear Plate® (Greiner Bio-One, Nürtingen, Germany) was loaded with GrowDex®-T
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44 (#200103010; UPM-Kymmene Corporation, Helsinki, Finland) pre-mixed with 800,000 PODS®-
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46 hBDNF (#PPH1-50; Cell Guidance Systems, Cambridge, UK) or 20 ng/ml of recombinant human
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48 BDNF (RhBDNF) (R&D system, Minneapolis, MN, USA). Spheroids were then carefully
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50 transferred from culture plates to a flat-bottom plate with direct injection into the GrowDex®-T
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52 solution. GrowDex®-T was diluted to 0.5% (w/v) and 0.375% (w/v) throughout the *in vitro* study
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4 based on the manufacturer's instructions. Anionic NFC hydrogel (GrowDex®-T) was kindly
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6 provided by the UPM-Kymmene Corporation (Helsinki, Finland). Cellulose kraft pulp was
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8 chemically modified and fibrillated to form anionic NFC hydrogels, the diameter of which
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10 ranged from 4–10 nm and lengths from 500–10,000 nm, measured using electron microscopy.
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14 PODS®-hBDNF was kindly provided by Cell Guidance System (Cambridge, UK). NeuroFluor™
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16 NeuO (#01801; STEMCELL Technologies, British Columbia, Canada) was used for the detection
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18 of live neurons.
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21 22 *2.3. Immunocytochemistry (monolayer culture)*

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25 Immunocytochemistry on cells cultured on a two-dimensional (2D) monolayer was
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27 performed based on our protocol that has been previously described [44]. See also the
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29 Supplemental Data in detail. The primary and secondary antibodies used in this study are listed
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31 in Supplementary Tables S1 and S2, respectively. Immunocytochemistry controls were also
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33 performed as previously described [45,46]. The primary antibody controls were performed with
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35 immunocytochemistry with the primary antibody and hESC-derived ONPs with a second
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37 primary antibody to the same antigen [45]. Secondary antibody controls were performed each
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39 time multiple primary antibodies were used [45]. Labeling controls (detection controls) were
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41 performed for a sample from each batch of hESC culture [45]. See Supplementary Fig. S1 for
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43 exemplary figures for these control conditions.
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50 51 *2.4. Immunocytochemistry (three-dimensional spheroid culture)*

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53 For hESC-derived late-stage ONP spheroids, culture media were first removed and
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55 spheroids were fixed with 4% (w/v) paraformaldehyde followed by blocking overnight in a
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57 solution composed of 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) and 0.1%
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4 Triton X-100 in Dulbecco's Phosphate-Buffered Saline (DPBS) (Corning, Life Sciences,
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7 Tewksbury, MA, USA). Each sample was incubated for three days at room temperature with the
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9 primary antibodies that are listed in Supplementary Table S3. This was followed by several PBS
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11 washes and incubation in the dark for three days at room temperature with Alexa Fluor 488 and
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13 647 (#ab150129, #ab150075; Abcam, Cambridge, MA, USA). Nuclei were stained with 4',6-
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15 diamidino-2-phenylindole (DAPI; #D1306; Thermo Fisher Scientific, Waltham, MA, USA) for 30
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17 minutes. Images were then assessed using a Nikon A1 confocal microscope (Nikon, Tokyo,
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19 Japan). ImageJ ver. 2.0.0-rc-69/1.52p. (National Institutes of Health, Bethesda, MA, USA) was
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21 used to quantify images. Further details on image acquisition and quantification of immuno-
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23 positive cells are described in the Supplementary Data.
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30 *2.5. Enzyme-linked immunosorbent assay for brain-derived neurotrophic factor*

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33 To measure the concentration of BDNF secreted from PODS® crystals, culture media of
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35 both a control and an experimental condition were collected at each time point and
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37 immediately stored at -80°C before running an enzyme-linked immunosorbent assay (ELISA).
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39 The same method was applied to measure the degradation of RhBDNF. All samples were
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41 quantified with a BDNF ELISA kit (#BGK23560, PeproTech, Rocky Hill, NJ, USA), and the results
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43 were analyzed with a Synergy HTX Multi-Mode Reader (BioTek, Winocski, VT, USA) at 450 nm
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45 wavelength as instructed from the kit.
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50 *2.6. Rheological characterization of nanofibrillar hydrogel*

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53 Rheological characterization was performed on an MCR302 Rheometer (Anton Paar,
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55 Graz, Austria) using a 25-mm diameter plate at 37°C. Each sample was dispensed on the
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57 preheated rheometer plate with a gap distance of 1 mm, and excess hydrogel was removed.
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The testing protocol was then applied to the sample. Each hydrogel sample was used for only one test.

2.7. Deafening and genotyping of diphtheria toxin receptor-positive mice

The Institutional Animal Care and Use Committee of Northwestern University Feinberg School of Medicine approved our experimental procedures (IACUC Protocol number: IS00011516), which also met US National Institutes of Health guidelines for animal care and use. The protocol was also reviewed and approved by the US Army Animal Care and Use Review Office. Both male (N=12) and female (N=14) DTR mice (age 3–6 months old) were used as hESC-derived ONP recipients. Ten wild type (WT), C57/BL6, mice were used as a control. We chose a deaf-animal model in the DTR mouse group and developed a colony from a contribution of DTR mice provided by Dr. W. Edwin Rubel (University of Washington, Seattle, WA, USA). The DTR mice were deafened at a young-mature age (P28–P35) using a single injection of DT at a dose of 25 ng/g (intramuscular injection), causing hair-cell degeneration [42,43,47]. For identification of DTR mice (*Pou4f3^{DTR/+}*) and WT mice (*Pou4f3^{+/+}*), a tail biopsy was collected and DNA was extracted using a DNA blood and tissue kit (Qiagen, Germantown, MD, USA). The target allele was amplified using a Mastercycler® nexus (Eppendorf, Hamburg, Germany) and the primer (0.4 μM) listed in the Supplemental Data. Also, see the detailed description of PCR in the Supplementary Data.

2.8. Auditory brain stem response

Hearing loss induced by DT injection was confirmed by auditory brain stem response (ABR) recordings before and after injection. DTR mice were anesthetized with 67 mg/kg of ketamine and 3.3 mg/kg of xylazine (intraperitoneal injection with additional dosing of

ketamine as needed). Both tone-burst and click-evoked ABRs were used to assess the sensitivity of the nerve to acoustic stimuli to confirm the efficacy of the DTR mouse model. Both stimulus control and averaging were performed using TestPoint (Cape Town, South Africa) as well as SmartEP (Intelligent Hearing Systems, Miami, FL, USA). A detailed description of the ABR parameters and settings is provided in the Supplementary Data. We performed tone-burst evoked ABR both on three DTR mice and four WT mice to thoroughly test the frequency-specific hearing threshold. Click-evoked ABR was performed on the remaining mice.

2.9. Transplantation of human embryonic stem cell-derived otic neuronal progenitors

Human ESC-derived ONPs were transplanted in left cochleae and right cochleae were used as a control condition. Meloxicam (2 mg/kg, subcutaneous injection) was given prior to surgery to minimize respiratory distress; follow-up doses (1mg/kg/day) were given for three days. Anesthesia was induced with isoflurane at 3–4% (reduced to 1–2% after induction), delivered with oxygen (0.3 L/min) and nitrous oxide (0.25 L/min) gas. The animals' heads were secured to a custom head-holder that delivered the anesthetic gas. Body temperature was maintained by a circulating water pad and insensate fluid loss replaced at 0.1 mL/10g (body weight). A post-auricular incision was made, and bone (caudal to the stylomastoid foramen) was removed with a surgical drill to reveal the round window. The round window membrane was then excised using a 30–33 G needle. Dissociated hESC-derived ONPs (250,000 cells) or hESC-derived ONP spheroids (five spheroids where each spheroid contains 50,000 ONPs) were delivered to the scala tympani by a glass micropipette while manipulating the pipette's pressure with a Xenoworks Digital Injector (Sutter Instrument, Navato, CA, USA). The pressure range used for a spheroid transplantation was between -1 hPa and +5 hPa. Two μ L of 1%

GrowDex®-T with 800,000 of PODS®-hBDNF was delivered using a 10-μL Hamilton syringe. After the transplantation was completed, the round window was covered by a small piece of fascia and secured with Vet Bond™ adhesive (3M, Minneapolis, MN, USA). Muscle and skin were closed in layers and the animal was allowed to recover with thermal therapy. The animal was kept for 90 days, allowing engraftment and neuronal differentiation in the inner ear. No immunosuppressant was administered postoperatively [18,48].

2.10. Immunohistochemistry

After completion of the post-implantation survival period, each animal was euthanized, the cochlea was dissected, and the specimen was sectioned into 10-μm slices on a Leica CM3050 S cryostat (Leica Inc., Nussloch, Germany). Immunohistochemistry was performed using standard techniques with a mouse anti-human nuclear antibody (AHNA) (1:100, STEM101, Takara Bio, Tokyo, Japan) and a rabbit anti-β-III tubulin antibody (1:500, Abcam, Cambridge, MA, USA). See detail in the Supplementary Data. Both positive and negative control conditions were obtained to ascertain the specificities of AHNA (Supplementary Fig. S2). Laser scanning confocal imagery was performed with a Leica TCS SP5 microscope (Leica, Inc., Nussloch, Germany).

2.11. Neurite bearing, neurite growth, and neurite arborization assays

A pan-neuronal marker, β-III tubulin was used to visualize neurite-bearing cells, measure neurite length, and calculate neurite arborization area. The analysis was performed by examining an individual confocal optical Z-stack section through the entire extent of each spheroid at the observation time point using ImageJ ver. 2.0.0-rc-69/1.52p (National Institute of Health, Bethesda, MD, USA) [49]. Due to the nature of baseline fluorescent signaling from the

hydrogel, the watershed algorithm was extensively used to identify an individual nucleus [50]. Neurite-bearing cells were quantified by counting the number of neurites in a confocal optical section with neurite lengths at least twice the diameter of the cell body. The ratio of neurite-bearing cells to the total number of cells was computed for each condition. The length of neurites was determined by manually tracing the length of the longest neurite on 20 randomly selected hESC-derived ONPs. For a neurite arborization assay, each Z-stack photomicrograph was transformed into an 8-bit grayscale [51,52]. Quantification was assessed after defining a threshold for background correction using ImageJ.

2.12. Statistical analysis

When appropriate, and as indicated in each figure, statistical analysis was performed. Three biological and technical replicates were obtained and used for statistical analysis unless otherwise noted [53,54]. Experimental values are typically expressed as mean $\pm$ standard error. All statistical analyses were performed with R (version 3.5.1. 2018-07-02, Vienna, Austria) [55]. Normal distributions were assumed. An analysis of variance (one-way) with Bonferroni-corrected *post hoc* t-tests or two-tailed t-tests were performed. P values smaller than 0.05 were considered statistically significant.

3. Results

3.1 Neuronal differentiation of human embryonic cell-derived otic neuronal progenitors with the polyhedrin delivery system

Human ESCs (H1, H7, and H9) were sequentially differentiated into late-stage ONPs using the protocol outlined in Fig. 1. Human ESC-derived late-stage ONPs were then treated with 800,000/ μ L of PODS®-hBDNF for seven days in NIM or BrainPhys™ (Fig. 2A). As compared

with late-stage ONPs (Fig. 2B), the treated cells contained more bipolar-shaped neurites, consistent with the morphology of human SGNs (Fig. 2C) [56]. After seven days of the treatment, late-stage ONPs highly expressed GATA3 (otic lineage marker) [57,58], Neurogenin-1 (NeuroG1, otic lineage marker) [59,60], PAX8 (otic lineage marker, more specifically otic placode and otic vesicle marker) [61,62], SOX2 (neuronal progenitor marker) [57], nestin (neuronal progenitor marker) [57], vesicular glutamate transporter-2 (VGLUT2, glutamatergic neuronal marker) [63], β -III tubulin (pan-neuronal marker) [64,65], and peripherin (peripheral sensory neuron marker) [66] (Fig. 2D-I). PODS[®]-hBDNF-treated ONPs did not express E-cadherin (ECAD, an otic epithelial marker) [67], PAX6 (central nervous system neuronal marker) [68], nor SOX10 (glial marker) [69] (data not shown), suggesting that the cells differentiated toward an otic neuronal lineage, rather than other lineages such as an otic-epithelial lineage, central nervous system neuronal lineage, or glial lineage.

With PODS[®]-hBDNF treatment of the late-stage ONPs, nestin and SOX2 were expressed significantly lower than other markers, suggesting that the treatment facilitated an otic neuronal differentiation (Fig. 2J). In the quantification of PAX8- and SOX2-positive cells, the PODS[®] treatment significantly decreased the positivity of nestin (from 71.3 % $\pm$ 3.42 to 41.0 % $\pm$ 4.35) and that of SOX2 (from 64.2% $\pm$ 2.35 to 45.2% $\pm$ 2.90) as compared to RhBDNF treatment, suggesting that PODS[®] treatment more effectively differentiated the late-stage ONPs into otic neuronal fates (Fig. 2K). However, there were no statistically significant differences in the expression of PAX8 between RhBDNF (99.6 % $\pm$ 0.294) and PODS[®]-hBDNF treatment (99.8% $\pm$ 0.222) (Fig. 2K).

3.2. The generation of human embryonic cell-derived otic neuronal progenitor spheroids

Human ESC-derived ONP spheroids were generated from dissociated late-stage ONPs using an EZSPHERE® or 96-well Clear Round Bottom Ultra-Low Attachment Microplate® (Fig. 3A–B). A live neuron assay was performed to evaluate the viability of an hESC-derived ONP spheroid cultured in NIM/BrainPhys™ after seven days. An hESC-derived ONP spheroid was labeled with 0.25 μ M of NeuroFluor™ NeuO and incubated for one hour (shown in green in Fig.3C), demonstrating that the spheroid was β -III tubulin positive [70]. A 3-D immunocytochemical analysis also indicated that hESC-derived ONP spheroids expressed the positivity of PAX8, β -III tubulin, and nestin when cultured in NIM/BrainPhys™ for seven days (Fig. 3D), suggesting that the spheroids were differentiating into an otic neuronal lineage.

3.3. Rheological and viscoelastic properties of neurofibrillary hydrogel

Before performing a 3-D culture of hESC-derived ONP spheroids with NFC hydrogel, the mechanical properties of the hydrogel were first rheologically characterized by frequency sweep testing (Fig. 4A). The frequency sweep in Fig. 4A demonstrates that previously reported characteristic behavior of NFC hydrogel in that the loss factor ($\tan \delta = G''/G'$) was much lower than 1 [29]. This finding demonstrates its gel-like structure. Also, note that this behavior is nearly independent of the frequency. In addition, 0.375% GrowDex®-T showed a higher loss factor ($\tan \delta = G''/G'$) over 0.5% and 1% GrowDex®-T. Viscosity over shear stress was also measured to optimize the concentration suitable for the injection of GrowDex®-T in that 1% GrowDex®-T showed higher viscosity over 0.5% and 0.375% GrowDex®-T in lower shear stress (Fig. 4B). At high-stress level (> 20 Pa, applicable for injections), it also exhibited more of a fluid-like behavior (Fig. 4B). Shear moduli (G) of different concentrations of GrowDex®-T were estimated to determine their rigidities. In Fig. 4C, 1% GrowDex®-T demonstrates a

disproportionally higher shear modulus than 0.5% and 0.375% GrowDex®-T above 7% of shear strain.

3.4. In vitro culture of human embryonic stem cell-derived otic neuronal progenitor spheroids with neurofibrillary hydrogel

After the rheological characterization, we cultured hESC-derived ONP spheroids with 0.375% GrowDex®-T for seven days. Fig. 4D shows a schematic diagram of this part of the experiment. Three-dimensional immunocytochemistry was then performed to assess the positivity of PAX8, β -III tubulin, MAP2 (neuronal cytoskeleton marker) [71,72], VGLUT2, and peripherin to show that hESC-derived ONP spheroids were expressing otic neuronal signals (Fig. 4E-G).

3.5. Enzyme-linked immunosorbent assay for brain-derived neurotrophic factor

Before the use of PODS®-hBDNF with our ONP spheroids culture, we quantified the release kinetics of PODS®-hBDNF using ELISA. The concentration we detected from the supernatant of NIM containing 800,000 of PODS®-hBDNF showed steady zero-order release kinetics of hBDNF from PODS®-hBDNF, with approximately 25-50 pg/mL per day (Fig. 5A-B). Although PODS®-hBDNF crystals were encased in different concentrations of GrowDex®-T (i.e., 0.5% vs. 0.375%), both groups showed similar release profiles, with a total amount of approximately 300 pg/mL over seven days (Fig. 5A-B). On the contrary, a one-time dosage of 20 ng/mL of RhBDNF provided a sufficient amount of hBDNF on the first day, but after three days, the active hBDNF left in both of the culture media had less than 100 pg/mL (Supplementary Figure S2). Finally, we evaluated endogenously secreted hBDNF from hESC-derived ONP spheroids. An ONP spheroid (the cell density of 50,000 cells/spheroid) was cultured in NIM

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4 without PODS®-hBDNF. There was virtually no BDNF detected in an NIM-only condition with the
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7 spheroids by ELISA (shown in green in Fig. 5A–B).

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9 *3.6. In vitro culture of human embryonic stem cell-derived otic neuronal progenitor spheroids*
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11 *with nanofibrillar hydrogel and a polyhedrin delivery system*
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14 Human ESC-derived ONP spheroids were further cultured with GrowDex®-T and PODS®-
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16 hBDNF for seven days. The schematic diagram in Fig. 6A shows this part of our experiment.
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18 Phase-contrasted images of hESC-derived ONP spheroids cultured in GrowDex®-T and PODS®-
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20 hBDNF demonstrate that the integrity of spheroids tended to decay more so with GrowDex®-T
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22 and PODS®-hBDNF than only with GrowDex®-T over 48–72 hours (Fig. 6B(a–c) vs. Fig. 6B(e–f)).
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24 The image also shows that the cells in the spheroids may have been migrating toward PODS®-
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26 hBDNF crystals, suggesting that a BDNF gradient may be attracting hESC-derived ONPs in a
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28 chemotactic fashion (the white arrow in Fig. 6B(f)). Fig. 6C–F and Fig. 7 A–D show
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30 immunocytochemistry of hESC-derived ONP spheroids cultured with 0.5% GrowDex®-T for
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32 seven days, and 0.375% of GrowDex®-T, respectively. As seen in Fig. 6B, hESC-derived ONP
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34 spheroids maintained the integrity of their spheroidal shape better when they were cultured
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36 without GrowDex®-T or PODS®-hBDNF (Fig. 6C).

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38 The quantification of PAX8 expression on immunocytochemistry demonstrates the
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40 advantages of GrowDex®-T and PODS®-hBDNF in otic neuronal differentiation. We chose PAX8
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42 expression to gauge the degree of otic neuronal differentiation as PAX8 expression is known to
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44 peak at the otic placode or otocyst stage (corresponding to the ONP-stage) [73,74] and then to
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46 downregulate afterward [73–75]. Fig. 7E demonstrates that PAX8 positive cells are significantly
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48 more common in NIM-only conditions ($97.7\% \pm 0.14$ for 0.5% GrowDex®-T + PODS®-hBDNF and
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99.0% $\pm$ 0.44 for 0.375% GrowDex®-T + PODS®-hBDNF; shown in green bars) as compared to other conditions. This finding suggests that GrowDex®-T in conjunction with PODS®-hBDNF can facilitate otic neuronal differentiation. This finding is also apparent in the second row of Fig. 6C–F and Fig. 7A–D in that the positivity of PAX 8 was most obvious in NIM only conditions.

The quantification of neurite lengths in hESC-derived ONP spheroids demonstrates significantly longer neurites in the spheroids that were cultured in the combination of GrowDex®-T and PODS®-hBDNF (Fig. 7F). The number of neurite-bearing cells in ONP spheroids was significantly smaller when they were cultured in an NIM-only condition (Fig. 7G). Finally, a similar trend was observed in our neurite arborization assay as in the neurite growth assay (Fig. 7F) in that axonal branching was significantly greater in a culture condition with GrowDex®-T and PODS®-hBDNF (Fig. 7H).

3.7. In vivo transplantation of human embryonic stem cell-derived otic neuronal progenitor spheroids into the inner ear

Fig. 8A shows the experimental paradigm of this part of the study. Before stem cell transplantation in the inner ear, both tone burst-evoked and click-evoked ABRs were used to assess the sensitivity of the inner ear to acoustic stimuli to confirm the efficacy of the DTR mouse model. Fig. 8A and 8B shows exemplary data of typical tone burst-evoked ABR waveforms on a WT C57/BL6 mouse and a deafened DTR mouse. A level- and frequency-series of tone-burst-evoked ABRs demonstrated that a DTR mouse had no detectable ABRs even at the lowest attenuation levels (i.e., 0 to -20 dB, equivalent to 100–120 dB SPL) (Fig. 8B). Fig. 8C shows exemplary data of click-evoked ABR waveforms for a WT mouse (Fig. 8C: left column) and a deafened DTR mouse (Fig. 8C: right column). Note that a deafened DTR mouse

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4 demonstrates no detectable wave I or V even with the highest stimulation levels, indicating that
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6 they were adequately deafened for the study. Fig. 8D shows plots of ABR threshold audiograms
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8 of three DTR mice injected with DT and of eight WT mice. For each DTR mouse, no ABRs were
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10 detected at any tested frequency.
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14 Human ESC-derived ONP spheroids were then transplanted into the DTR mouse cochlea.
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16 First, dissociated hESC-derived ONPs were transplanted with NIM + 10 ng/mL of RhBDNF in a
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18 DTR mouse cochlea. Fig. 9A(a) shows that no apparent transplanted ONPs survived in the inner
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20 ear 90 days after the transplantation. Clear demarcation of the population of extant SGNs was
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22 noted by β -III tubulin staining. Dissociated ONPs were also transplanted with 1% GrowDex[®]-T
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24 with and without PODS[®]-RhBDNF. Fig. 9A (b-c) show that transplanted cells were engrafted in
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26 the scala media and lodged onto the organ of Corti (Fig. 9A(b)), with scar tissue formation in the
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28 scala media (note triple-positive cells for TOTO3, β -III tubulin, and AHNA). Human ESC-derived
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30 ONP spheroids were also transplanted into the inner ear with 1% GrowDex[®]-T with and without
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32 PODS[®]-hBDNF. After 90 days, transplanted cells were engrafted in the scala media (Fig. 9B(a))
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34 and in the scala tympani (Fig. 9B(b-c)). Note that as Fig. 9B shows, the transplanted cells still
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36 maintained an aggregated form in the inner ear. Finally, Fig. 9B(c-d) demonstrates that hESC-
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38 derived spheroids lodged in the wall of the scala tympani, extending neurites toward the
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40 septum between the scala vestibuli and the scala tympani. We then performed quantification
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42 of engrafted ONPs (triple-positive cells for TOTO3, β -III tubulin, and AHNA). Engraftment was
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44 significantly better with GrowDex[®]-T and GrowDex[®]-T with PODS[®]-hBDNF (Fig. 9C). Further
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46 quantitative analysis confirmed that GrowDex[®]-T with PODS[®]-hBDNF promoted neurite
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48 extension of engrafted hESC-derived ONPs *in vivo* (Fig. 9D).
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Discussion

The number and integrity of surviving SGNs have been implicated as playing an essential role in hearing sensitivity for deafened patients reliant on a cochlear implant or future stem-cell replacement therapy. A sustained ability to repopulate SGNs would enable improved clinical results [76–80]. Moreover, while HC regeneration is an important research area, successful inner ear function will still depend on the effective transmission of an electrical signal to the brainstem via SGNs. Therefore, novel techniques to enhance the efficiency of stem cell-derived ONP transplantation to augment the extant SGN population are critical. Historically, the low overall survival of transplanted stem cell-derived ONPs into the inner ear can be attributed to the relatively inhospitable environment of the bony inner ear and inadequate levels of trophic factor [18,48,81,82]. To overcome these issues, we artificially created a stem cell niche using hESC-derived ONP spheroids, NFC hydrogel, and a self-sustaining source of BDNF to promote survival and neuronal differentiation of transplanted hESC-derived ONPs.

Immunocytochemistry on our monolayer culture revealed that hESC-derived ONPs that were cultured with PODS®-hBDNF for seven days expressed adequate markers for human SGNs, consistent with our previously reported data [44,57,74,81]. In addition, PODS®-hBDNF treatment on these cells showed that it promoted otic neuronal differentiation as it significantly decreased nestin- and SOX2- positive cells; however, PAX8-positive cells did not significantly differ between RhBDNF treatment and PODS®-hBDNF treatment. Our immunocytochemistry data on hESC-derived ONP spheroids, however, indicated significantly decreased PAX8-positive cells in PODS®-hBDNF and GrowDex®-T, suggesting that an artificial stem cell niche generated

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4 by a combination of 3D-spheroidal structure, NFC hydrogel, and PODS®-hBDNF promoted
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6 further otic neuronal differentiation than a 2-D monolayer ONP culture with PODS®-hBDNF.
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10 We performed a rheological analysis of NFC hydrogel for two reasons. 1) the GrowDex®-
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12 T we used in this study is an anionic-charged NFC hydrogel which is different from GrowDex®
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14 (cationic-charged NFC hydrogel) [29]. The rheological characteristics of NFC hydrogel, including
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16 swelling properties, can be affected by the extent to which the hydrogel is ionized. 2) the
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18 original concentration of GrowDex®-T was 1%; however, microscopical visualization of cells in
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20 1% GrowDex®-T was extremely challenging, and we therefore decided to dilute it to 0.5% and
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22 0.375%, dilutions for which rheological characteristics had to be determined. The grow
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24 frequency sweep in NFC hydrogels demonstrated that all the NFC hydrogel samples had
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26 approximately $\tan\delta$ (G''/G') $\sim$ 0.08-0.13 (gel-like structure) and a little frequency dependence,
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28 which is usually an indication of a self-standing elastic gel. Influence of shear stress on the
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30 viscosity of 1% and 0.5% NFC hydrogels indicated reversible gelation in that fluid-like behavior
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32 was observed at high shear stress levels (> 10 Pa for 0.5% and > 20 Pa for 1% NFC hydrogel)
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34 whereas gel-like behavior was observed at low shear stress levels. This finding is consistent with
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36 previous studies that used NFC hydrogel as well as other physiological hydrogels [29,31,83,84].
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38 However, 0.375% NFC hydrogel did not demonstrate this feature; presumably because of the
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40 high water content of 0.375% NFC hydrogel.
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51 A strain sweep in NFC hydrogels indicated that both 0.375% and 0.5% NFC hydrogels
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53 showed nearly linear deformation in the entire measured strain regions (1–15%) due to their
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55 higher portion of water content. However, 1% NFC hydrogel deviated from linearity above 7%
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57 of shear strain. This disproportionally higher shear modulus shows that the yield stress was not
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4 directly proportional to the concentration of the 1% hydrogel above 7% of shear strain. This
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6 finding can be attributed to the composition of 1% hydrogel contains more cellulose, which
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8 formed fiber-like aggregates when higher shear strain was applied. This kind of “shear thinning”
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10 behavior of the NFC hydrogel made it an attractive ECM for our clinical application as the
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12 behavior allows the encasing of hESCs in the gel in a low shear force environment. When it
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14 comes to injections (i.e., high shear force) in the inner ear, NFC hydrogel allows for smooth
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16 injections with a syringe into the inner ear. We chose 1% NFC hydrogel for injections in our in
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18 vivo experiments to utilize its shear thinning behavior.
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25 The therapeutic importance of BDNF in the inner-ear regeneration has led to many
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27 studies exploring its therapeutic potential of BDNF; however, various studies have indicated
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29 that the half-life of BDNF is 10–30 mins and that this feature significantly limits its therapeutic
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31 potential [85,86]. Our ELISA result showed a steady rate of release of RhBDNF from PODS®
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33 crystals at approximately 25–50 pg/mL per day. With this rate of provision of RhBDNF, *in vitro*
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35 and *in vivo* immunocytochemistry demonstrated the promotion of otic neuronal differentiation
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37 and enhanced axonal growth on transplanted ONPs as compared to a condition with 20 ng/mL
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39 of RhBDNF. It is worth mentioning here that the majority of previous studies on hESC-derived
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41 ONP transplantation in the inner ear used 10–20 ng/mL of RhBDNF in the culture media
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43 [11,20,81]. The use of this particular concentration of BDNF was based on a study by Shi et al.
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45 that found the similar effect of BDNF on the induction of peripherin within the range of 1–100
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47 ng/mL [87]. Our in vitro and in vivo results support the notion that with the use of PODS®-
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49 hBDNF, BDNF concentrations of 25–50 pg/mL were sufficient for hESC-derived ONPs to
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51 differentiate into an otic neuronal lineage.
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4 Notably, the endogenous secretion of BDNF from hESC-derived ONPs was not detected
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7 by ELISA. This is either because hESC-derived ONPs did not in fact secrete BDNF or the ONPs
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9 themselves consumed BDNF. In other words, our result of the release of 25–50 ng/mL of BDNF
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11 per day might have been greater because the ELISA measurement did not consider the BDNF
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13 that was consumed by BDNF-consuming cells (i.e., hESC-derived ONPs). While the exogenous
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15 provision of hBDNF with the polyhedrin delivery system promoted otic neuronal differentiation
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17 in our study, the degradation kinetics of PODS® crystals by any protease and long-term
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19 sequelae after more than 90 days is still undetermined. Further studies will be needed to
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21 investigate the endogenous provision of BDNF from hESC-derived ONPs.
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28 Multicellular spheroids consist of a viable cell rim and central necrosis, which typically
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30 arises at the spheroid diameter of 400–600 μm [88]. This was determined by fluorescence
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32 microscopy. The results of our hESC-derived ONP spheroids lacked the fluorescence signal in
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34 the center (Fig. 6-7). There are two possible explanations: 1) a limitation of the blood supply or
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36 of removal of the waste product caused by hypoxia [88] or 2) the lack of fluorescent antibody
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38 penetration to the core. Further studies using the hypoxia assay will be required to shed light
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40 on this matter. If it is, in fact, the latter case, mixing a low number of PODS®-hBDNF crystals at
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42 the time of ONP spheroid generation or reducing the total number of cells in one spheroid can
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44 be considered as methods to reduce the size of the central necrosis.
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52 Previous studies have shown that hESC-based stem cell therapy in the inner ear has
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54 been hindered by poor engraftment [18,20,81]. The majority of transplanted, dissociated hESCs
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56 were either unable to attach onto the lining of fluid-filled inner ear scalae or, even if they
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58 attached, the lack of aggregation initiated an apoptosis cascade, resulting in cell death [20,81].
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4 After our *in vivo* hESC-derived ONP transplantation, ONPs were found in the various scalae, still
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6 maintaining an aggregated formation, presumably because of 1) the 3D nature of the
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8 transplanted ONP spheroids, 2) the NFC hydrogel facilitated their attachment to the lining of
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10 scalae, and 3) the continuous release of BDNF through a polyhedrin delivery system. The
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12 provision of an artificial stem cell niche did improve the survival rate of our transplanted ONPs;
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14 however, we were not able to assess how many hESC-derived ONPs survived in the cochlea
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16 because of the undetermined condition of the central cores of spheroids. Once again, future
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18 studies assessing the lack of fluorescent signal at the cores of spheroids will shed light on this
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20 matter.
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27 On a technical note, hESC-derived ONP spheroid injections without the use of NFC
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29 hydrogel was not possible due to the constant backflow of perilymph from the scala tympani in
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31 the mouse. The use of 0.375% or 0.5% of GrowDex®-T for injections did not allow any ONP
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33 spheroid to advance further in the scala tympani, presumably due to low viscosity and the
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35 stiffness of the gel. The use of 1% GrowDex®-T was chosen because its high viscosity and
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37 stiffness make injections feasible, and also it allowed us to advance our hESC-derived ONPs
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39 further down in the scala tympani toward the mid-turn to secure the placement. The high
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41 viscosity and high stiffness of 1% GrowDex®-T also displaced some of the transplanted ONPs in
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43 the scala media or in the scala vestibuli. A further study may need to adjust the suitable
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45 stiffness and viscosity of NFC hydrogel so that a spheroid can be placed despite the constant
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47 perilymphatic outflow and so that a spheroid can remain localized in the scala tympani.
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56 We used the DTR mouse model for this study to create HC damage, not SGN damage in
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58 the bony modiolus. Surgical access to the bony modiolus poses significant risks of cerebrospinal
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fluid leaks and of damaging modiolar blood vessels [10,21]. These complications can result in meningitis and inner-ear ischemic stroke. Instead, our long-term goal is realizing a next generation “biohybrid” CI that combines the proven CI approach with hESC-replacement therapy [89]. Such hybrid devices are more practical than stem-cell-only approaches as the latter must overcome the additional challenge of restoring HCs. In our study, transplanted hESC-derived ONP spheroids lodged onto the epithelial layer of the scala tympani, extending neurites into the bony wall (Fig. 9B(d)). Although preliminary, this finding suggests that the artificial stem cell niches and local neuro-protective and reparative properties of microglia and macrophage initiated by DT deafening can potentially re-create a neuronal network between CI electrodes and extant SGNs in the future [90].

5. Conclusions

Taken all together, our data demonstrated that the transplanted hESC-derived ONP spheroids survived and neuronally differentiated into an otic neuronal lineage and extended neurites toward the bony wall of the cochlea. Our data *in vitro* and *in vivo* presented here provided sufficient evidence that we have established a robust, reproducible protocol for *in vivo* transplantation of hESC-derived ONPs to the inner ear. While a previous study has demonstrated the functional restoration of deafened gerbils’ hearing using dissociated hESC-derived ONPs, this study has not been replicable [11]. Using our protocol to create an artificial stem cell niche in the inner ear using a 3-D spheroid formation, NFC gel for ECM, and a polyhedrin delivery system for the continuous provision of BDNF, it is now possible once again to work on integrating transplanted hESC-derived ONPs further into the inner ear as well as to work toward achieving functional auditory neurons generated from hESCs.

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7. Author Disclosure Statement

All of the authors have no commercial association that might create a conflict of interest in connection with this work.

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Figure legends

Figure 1. Illustrative summary of a stepwise treatment protocol for deriving an otic neuronal lineage from undifferentiated H1, H7, and H9 human embryonic stem cells lines. Developmental stages are shown at the top, above a timeline, and key treatments are shown below, using color-coded bars. Abbreviations: D: days; NNE: nonneuronal ectoderm; PPE: preplacodal ectoderm; ONP: otic neuronal progenitor; BMP4: bone morphogenetic protein 4; SHH: Sonic hedgehog; ATRA: all-trans retinoic acid; EGF: epidermal growth factor; BDNF: brain-derived neurotrophic factor; NT3: neurotrophin 3; IGF-1: insulin-like growth factor 1; FGF2: fibroblast growth factor 2; CDM: chemically-defined medium; NIM: neural induction medium; MACS: magnetic-activated cell sorting; FACS: fluorescence-activated cell sorting; p75: low-affinity neurotrophin receptor (p75^{NTR}). This protocol is adapted from Matsuoka *et. al.*, 2017 and 2018.

Figure 2. Assessment of induction of an otic neuronal lineage from late-stage ONPs. **(A):** A stepwise treatment for an otic neuronal lineage induction. On day 25, PODS[®]-hBDNF treatment was started in NIM for 7 days. D: days. **(B):** Phase-contrast photomicrographs of hESC-derived late-stage ONPs. **(C):** Phase-contrast photomicrographs of hESC-derived ONPs treated with PODS[®]-hBDNF for seven days. **(D)(E)(F)(G)(H)(I):** Immunocytochemistry of hESC-derived late-stage ONPs treated with PODS[®]-hBDNF in NIM/Brainphys™ shows expression of various otic neuronal markers: GATA3, NEUROG1, PAX8, SOX2, nestin, VGLUT2, β -III tubulin, and peripherin. **(J):** Quantification of the otic neuronal markers for % positivity (n = 3) on hESC-derived late-stage ONPs treated with PODS[®]-hBDNF for seven days. **(K):** Quantification of % positivity for

nestin, PAX8, and SOX2 on PODS[®]-hBDNF and RhBDNF treated cells. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA with Tukey's *post-hoc* test. n.s.: not statistically significant.

Figure 3. Generation of hESC-derived ONP spheroids. **(A):** A schematic diagram of forming hESC-derived late-stage ONP spheroids using an EZSHPERE[®] plate (upper row) and a phase-contrast photomicrograph of individual spheroids (see white arrow) within the plate (bottom row). **(B):** A schematic diagram of forming hESC-derived ONP spheroids using a 96-well Clear Round Bottom Ultra-Low Attachment Microplate[®] (upper row) and a low-power (left bottom row) and high-power (right bottom row) phase-contrast photomicrograph of a spheroid within the plate. **(C):** A phase-contrast (upper row) and an epifluorescence (bottom row) photomicrographic image of a hESC-derived ONP spheroid stained with NeuroFluorT^M NeuO. **(D):** Immunocytochemistry on a hESC-derived ONP spheroid that was cultured for seven days with 800,000 of PODS[®]-hBDNF. The spheroid was stained for PAX8, β -III tubulin, and nestin.

Figure 4. Rheological characterization of GrowDex[®]-T and hESCs-derived ONPs spheroids culture with GrowDex[®]-T. **(A):** Frequency sweep (viscoelasticity) of three different concentrations (1%, 0.5%, and 0.375%) of GrowDex[®]-T (n=3). **(B):** Influence of shear stress on the viscosity of three different concentrations (1%, 0.5%, and 0.375%) of GrowDex[®]-T (n=3). **(C):** Strain sweep (stress-strain curve) of three different concentrations (1%, 0.5% and 0.375%) of GrowDex[®]-T. Shear modulus of 1%, 0.5% and 0.375% GrowDex[®]-T is plotted as a function of shear strain (n=3). **(D):** Schematic figure of hESC-derived ONP spheroids culture in GrowDex[®]-T.

(E), (F), and (G): Neuronal marker expressions of PAX8, β -III tubulin, MAP2, VGLUT2, peripherin and phase-contrast of hESC-derived ONP spheroids cultured with 0.375% GrowDex[®]-T.

Figure 5. Accumulative release kinetics of BDNF from PODS[®]-hBDNF and from a hESC-derived ONP spheroid cultured in NIM. **(A):** BDNF release profile of 800,000 PODS[®]-hBDNF in 0.5% GrowDex[®]-T over seven days. **(B):** BDNF release profile of 800,000 PODS[®]-hBDNF in 0.375% GrowDex[®]-T over seven days. In both **(A)** and **(B)**, accumulative release kinetic of BDNF from a hESC-derived ONP spheroid cultured in NIM is also plotted in a green line as a control.

Figure 6. Immunocytochemistry of hESC-derived ONP spheroids that were cultured with 0.5% GrowDex[®]-T *in vitro*. **(A):** Schematic diagram of PODS[®]-hBDNF and GrowDex[®]-T mixture with hESCs derived ONPs spheroids *in vitro*. **(B):** A phase-contrast photomicrograph of hESC-derived ONPs spheroids cultured with GrowDex[®]-T (gray background) and/or PODS[®]-hBDNF (shown in black). Human ESC-derived ONP spheroids that were cultured with 1% GrowDex[®]-T in for 24 hours (a), 48 hours (b), and 72 hours (c), respectively. Human ESC-derived ONP spheroids that were cultured with 1% GrowDex[®]-T and 800,000/ μ L of PODS[®]-hBDNF for 24 hours (d), 48 hours (e), and 72 hours (f), respectively. **(C):** Immunocytochemistry of hESC-derived ONP spheroids culture in NIM for 7 days. **(D):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.5% GrowDex[®]-T for seven days. **(E):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.5% GrowDex[®]-T with 20 ng/mL of RhBDNF for seven days. **(F):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.5% GrowDex[®]-T with 800,000/ μ L of PODS[®]-hBDNF for 7 days.

Figure 7. Immunocytochemistry of hESC-derived ONP spheroids cultured with 0.375% GrowDex[®]-T *in vitro*. **(A):** Immunocytochemistry of hESCs-derived ONPs spheroids cultured in NIM with 10 ng/nL of RhBDNF for seven days. **(B):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.375% GrowDex[®]-T for seven days. **(C):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.375% GrowDex[®]-T with 20 ng/mL of RhBDNF for seven days. **(D):** Immunocytochemistry of hESC-derived ONP spheroids cultured in 0.375% GrowDex[®]-T with 800,000/ μ L of PODS[®]-hBDNF for seven days. **(E):** Quantification of PAX8 positivity based on immunocytochemistry in hESC-derived ONP spheroids. **(F):** Quantification of neurite length arising from hESC-derived ONP spheroids (n=3). **(G):** Quantification of neurite bearing analysis. **(H):** Quantification of neurite arborization analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Tukey's *post-hoc* test.

Figure 8. *In vivo* hESC-derived ONP spheroid transplantation in the DTR mouse cochlea. **(A):** A schematic diagram of experimental design for *in vivo* hESC-derived ONP spheroid transplantation. **(B):** Tone-burst evoked ABR assessment of a WT mouse. **(C):** Tone-burst evoked ABR assessment of a DTR mouse. **(D):** Click-evoked ABR assessment of WT mouse (left column) and DTR mouse (right column). **(E):** ABR audiograms for 3 DTR and 8 WT mouse ears, with mean values for the latter shown with the gray line. Levels are expressed in terms of attenuation, with sound pressure levels at 0 dB ranging from 105 to 120 dB SPL. Thresholds were based upon visual detection of a response above the noise floor. No ABRs were detected for the three DTR mice (data plotted above the 0 dB line).

Figure 9. Immunohistochemistry (TOTO3 (counter staining), β -III tubulin, and AHNA) of transplanted hESC-derived ONPs in the DTR mouse cochlea. **(A):** Immunohistochemistry of a 10- μ m sliced DTR mouse cochlea transplanted with dissociated hESC-derived ONPs (250,000 cells). (a) Dissociated hESC-derived ONPs transplanted with NIM and 10 ng/mL of RhDBDNF. (b) Dissociated hESC-derived ONPs transplanted with 1% GrowDex[®]-T. (c) Dissociated hESC-derived ONPs transplanted with 1% GrowDex[®]-T and 800,000 of PODS[®]-hBDNF. White arrow in (b) and (c): Triple-positively stained cells with TOTO3, β -III tubulin, and AHNA. **(B):** Immunohistochemistry of (TOTO3 (counter staining), β -III tubulin, and AHNA) a 10- μ m sliced DTR mouse cochlea transplanted with hESC-derived ONP spheroids (5 spheroids/mouse). (a) HESC-derived ONP spheroids transplanted with 1% GrowDex[®]-T. b) Human ESC-derived ONP spheroids transplanted with 1% GrowDex[®]-T and 800,000 of PODS[®]-hBDNF. c) Another hESC-derived ONP spheroids transplanted with 1% GrowDex[®]-T and 800,000 of PODS[®]-hBDNF. White arrow in (b) and (c): triple-positive cells stained with TOTO3, β -III tubulin, and AHNA. d) High power magnification of the triple-positive cells in (c). Small white arrow: neurites. SM: scala media, SV: scala vestibuli, ST: scala tympani, S: scar tissue, RM: the Reissner's membrane, SGN, spiral ganglion neurons, HT: helicotrema, MO: modiolus. **(C):** Quantification of the number of the triple positive cells in dissociated ONPs transplantation vs. ONP spheroids transplantation. **(D):** Quantification of the number of neurites in dissociated ONPs transplantation vs. ONP spheroids transplantation. NIM: neuronal induction media, G: GrowDex[®]-T, G+P: GrowDex[®]-T and PODS[®]-hBDNF. * $p < 0.05$, ** $p < 0.01$, n.s.: not significant by one-way ANOVA with Tukey's

post-hoc test. Note that all of the images stained for TOTO3 iodide and AHNA have been pseudo-colored.

The diagram shows a vertical sequence of five stages of cell development, connected by upward-pointing arrows. The stages are:

- Stage 1 (Bottom):** A cluster of several small, rounded cells, each with a prominent nucleus.
- Stage 2:** A group of four cells that are more elongated and spindle-shaped than the first stage.
- Stage 3:** A group of four cells that are highly elongated and spindle-shaped, with a more complex internal structure.
- Stage 4:** A single, highly elongated and spindle-shaped cell, representing a specialized cell type.
- Stage 5 (Top):** A large, irregularly shaped mass of cells, representing a tissue or organ.

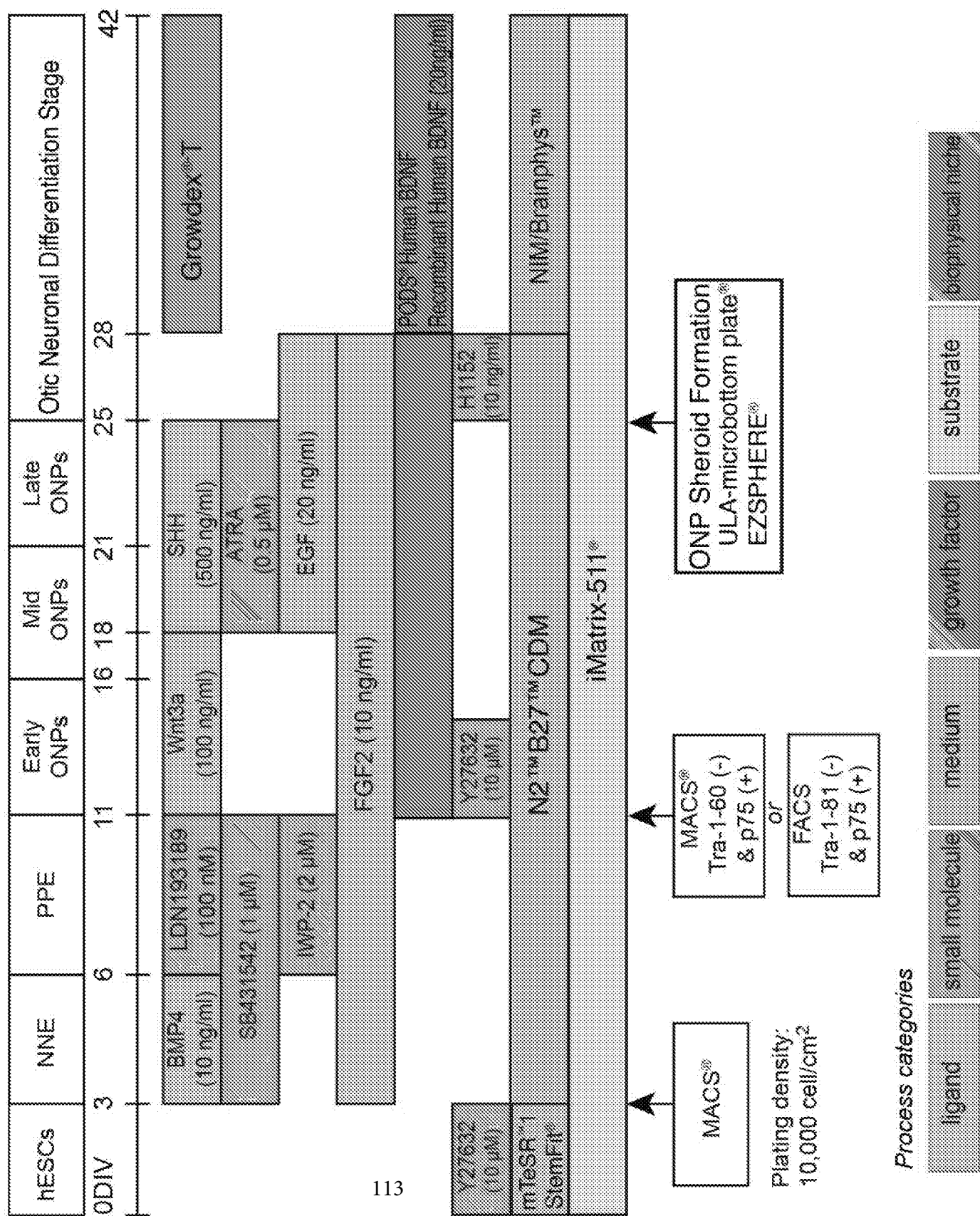


Figure 2

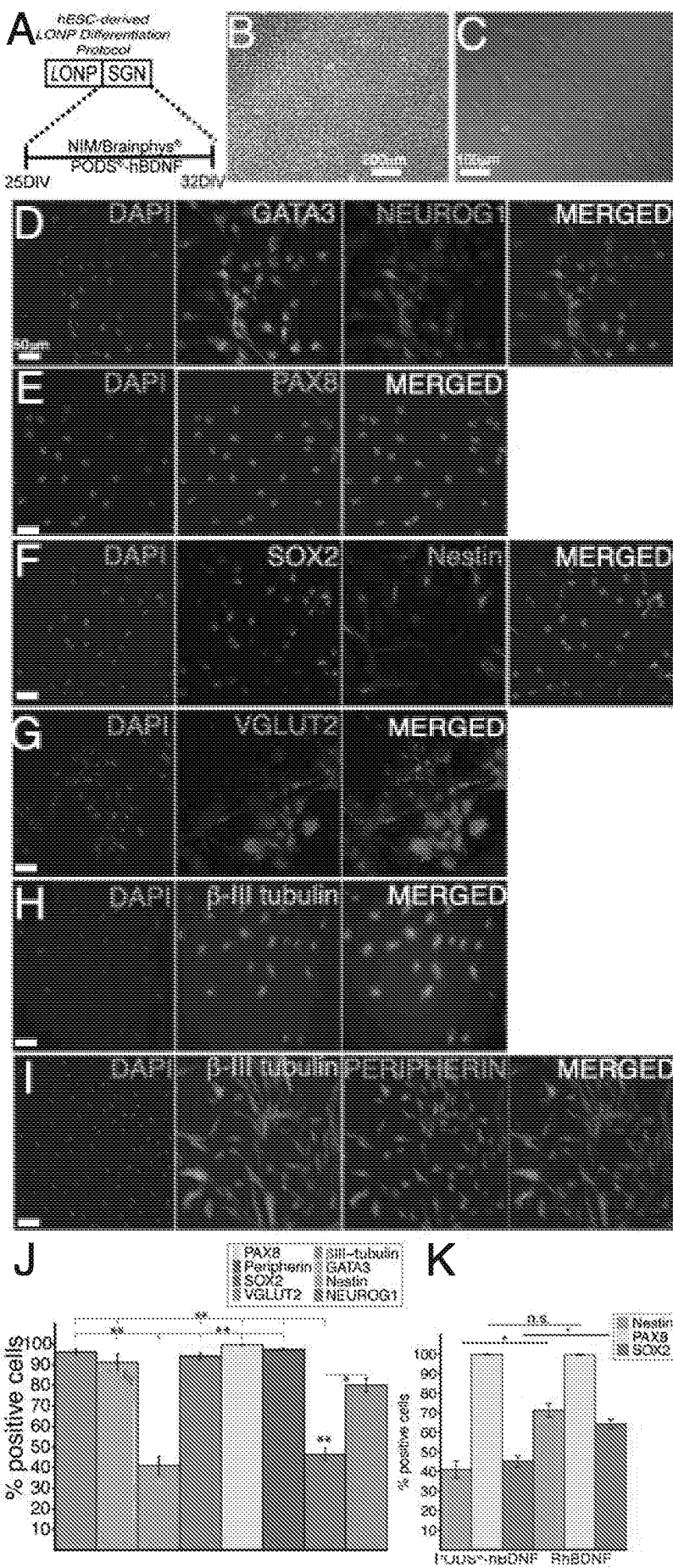


Figure 3

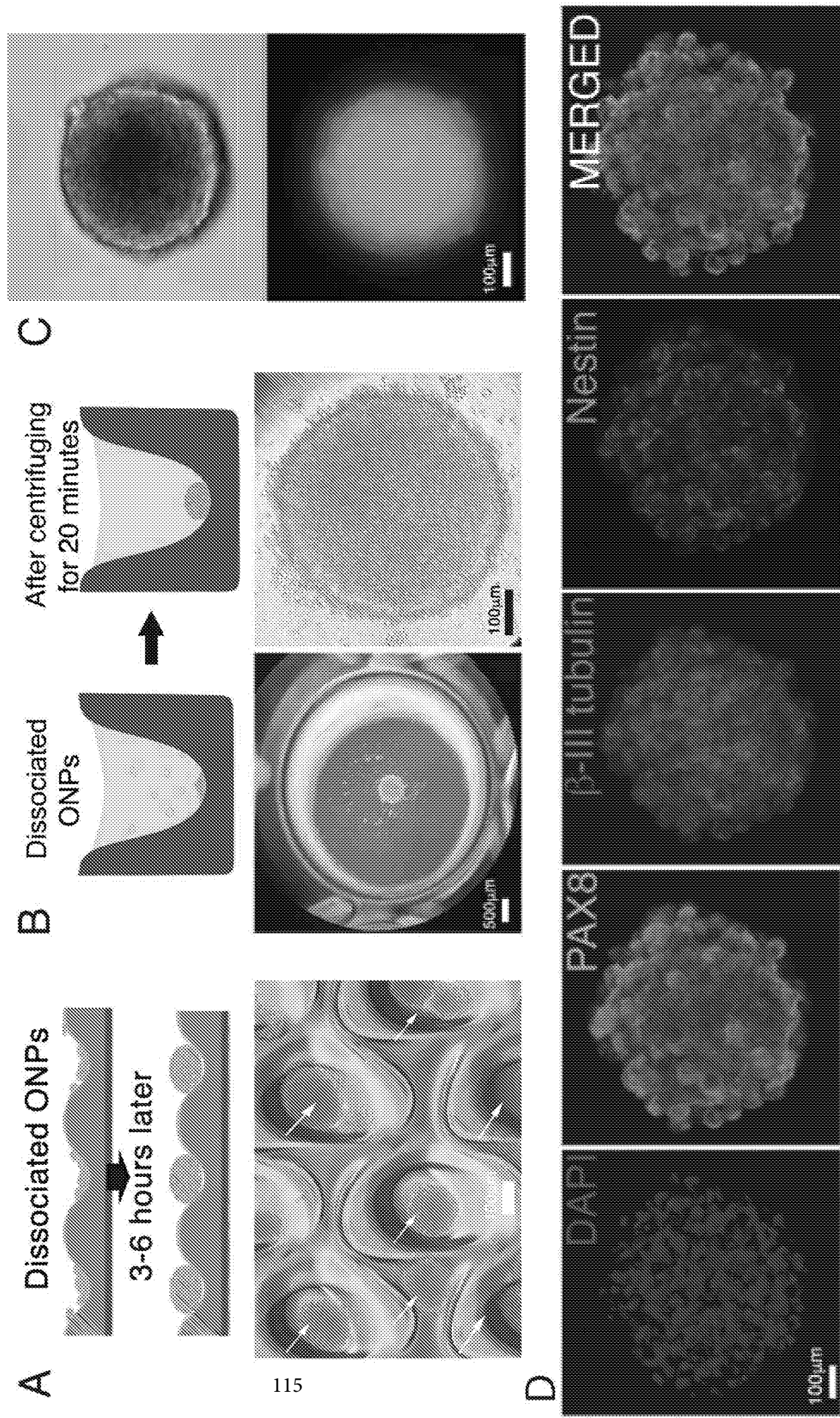
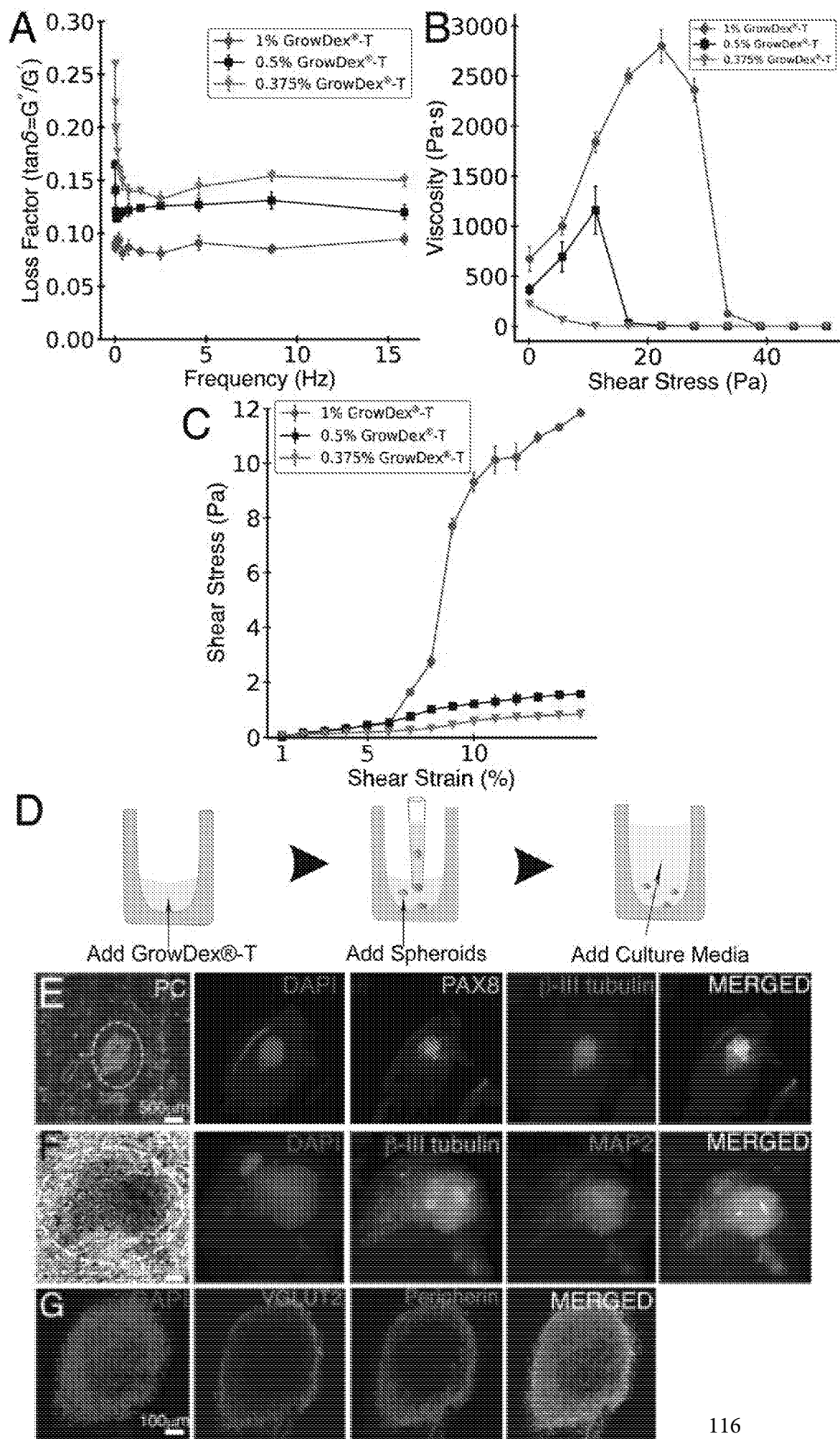


Figure 4



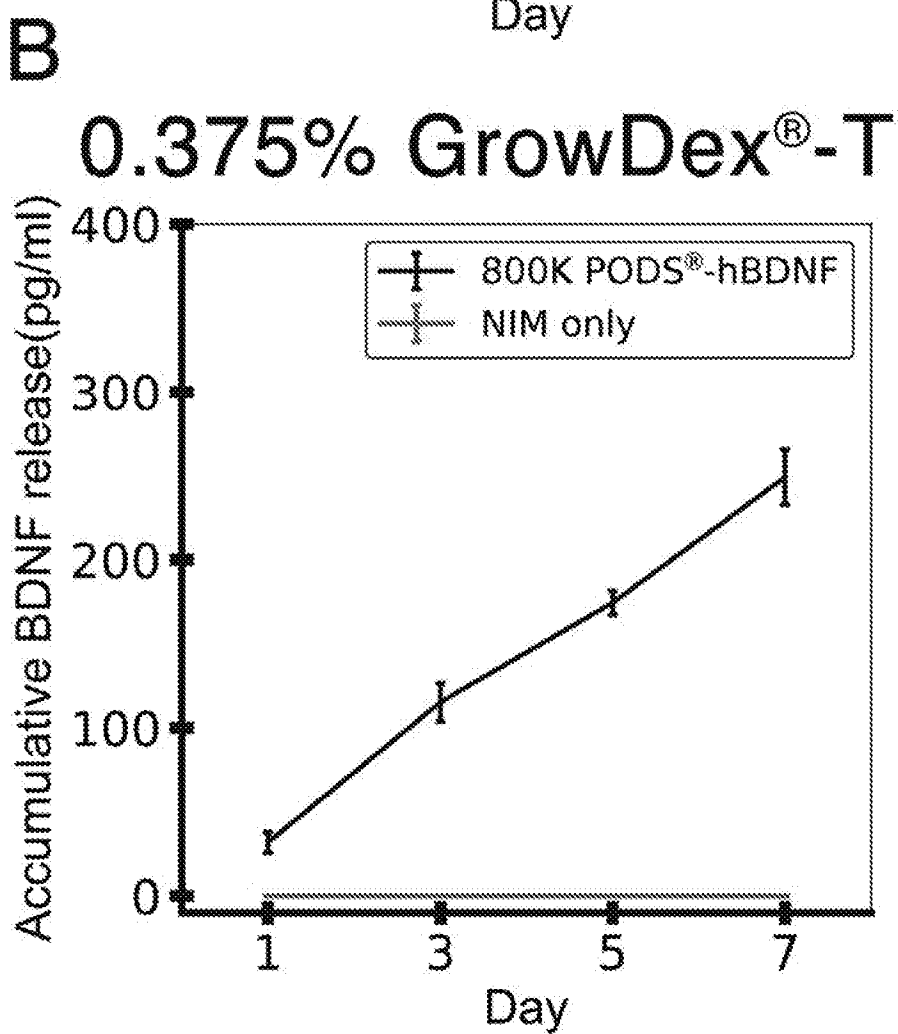
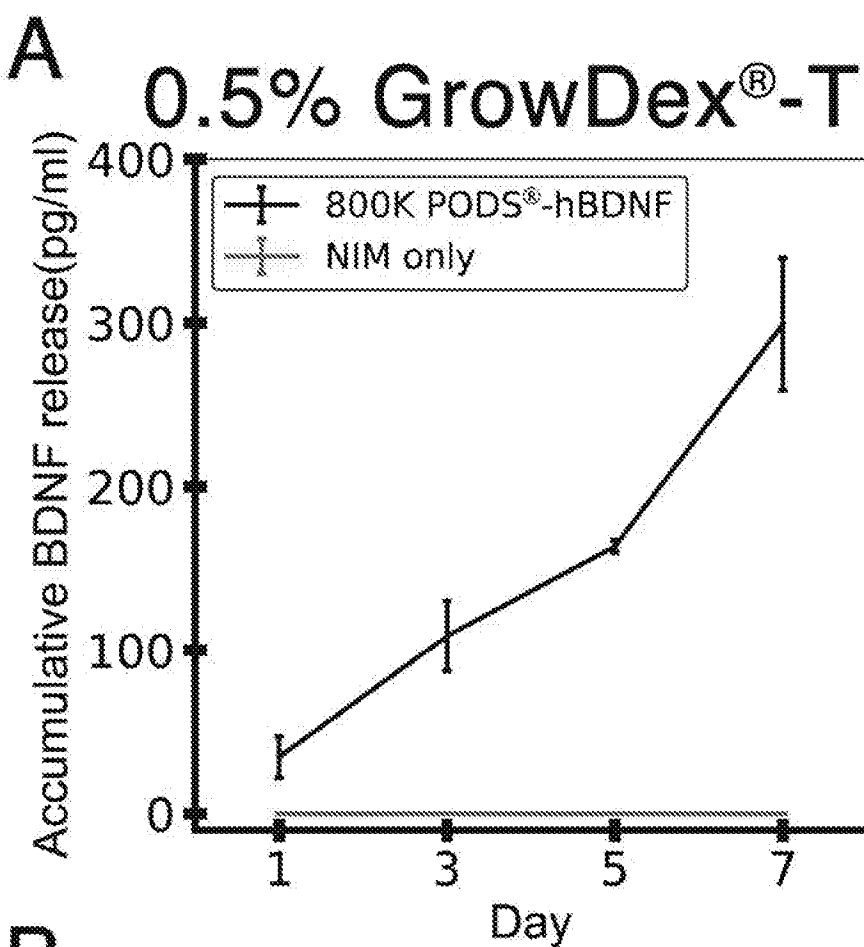


Figure 6

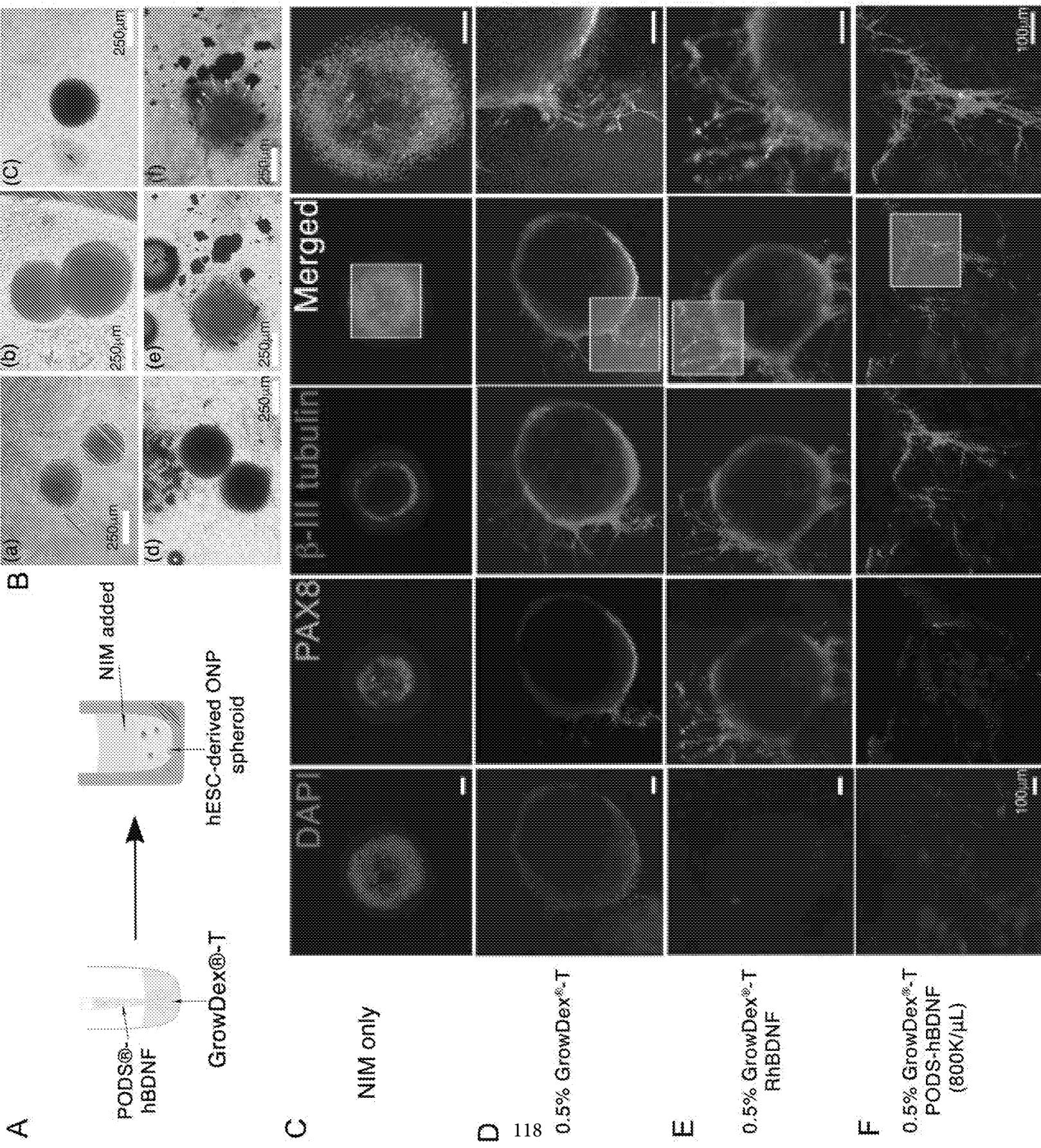


Figure 7

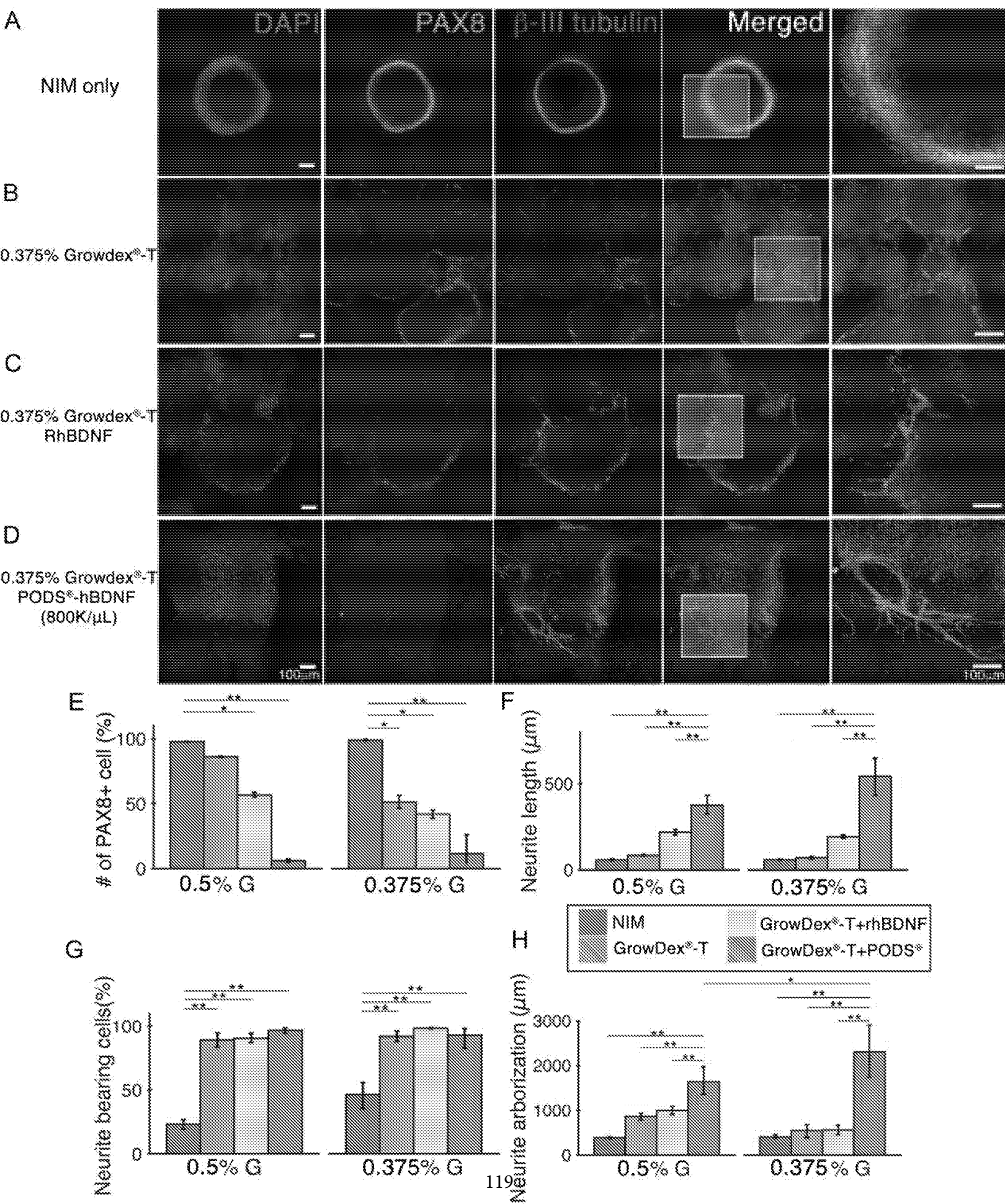
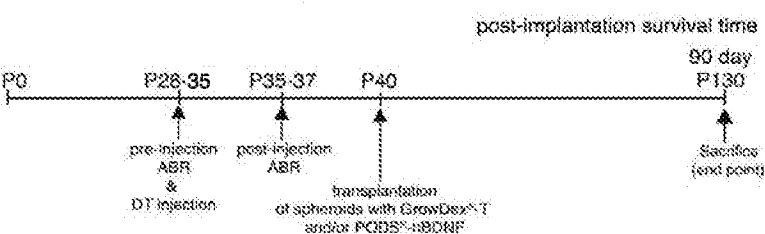


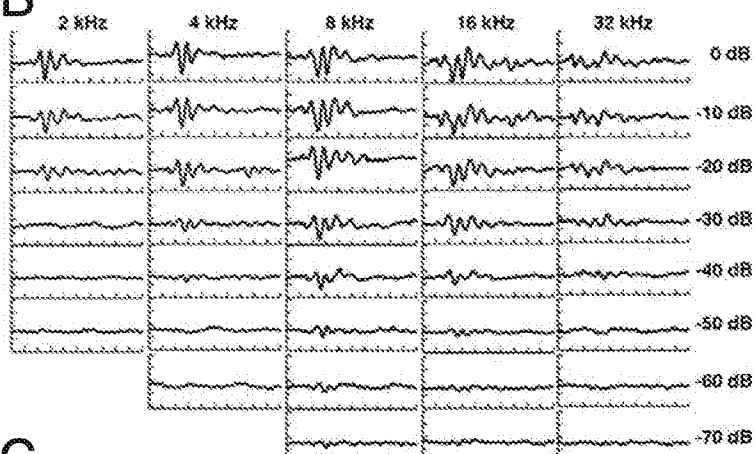
Figure 8

A

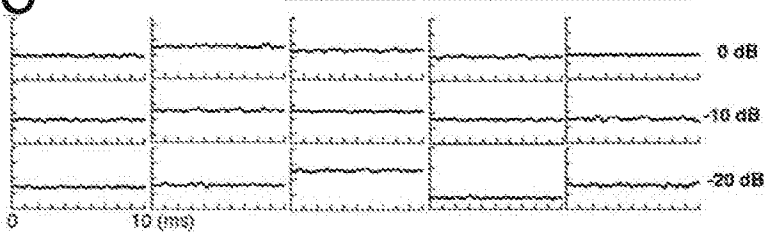
*hESC-derived 3-D spheroids
transplantation in DTR⁺ mice*



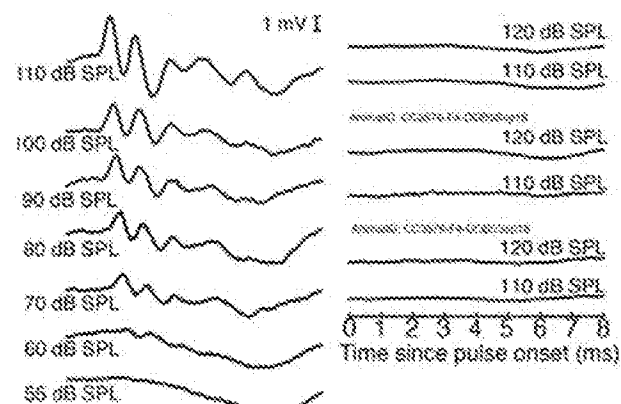
B



C



D



E

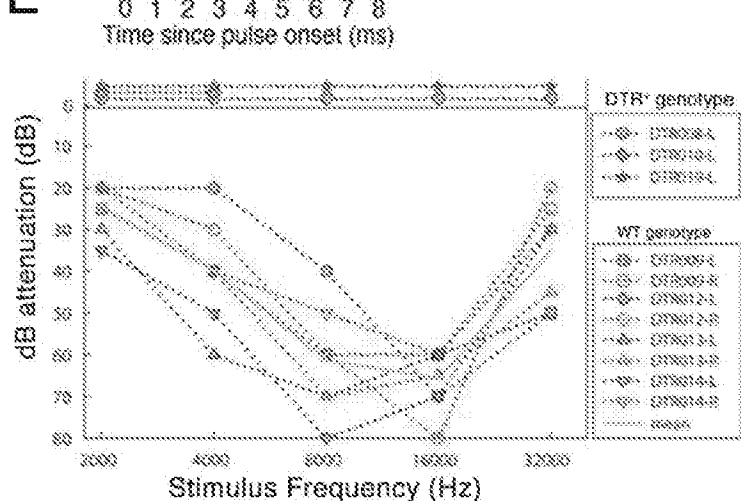
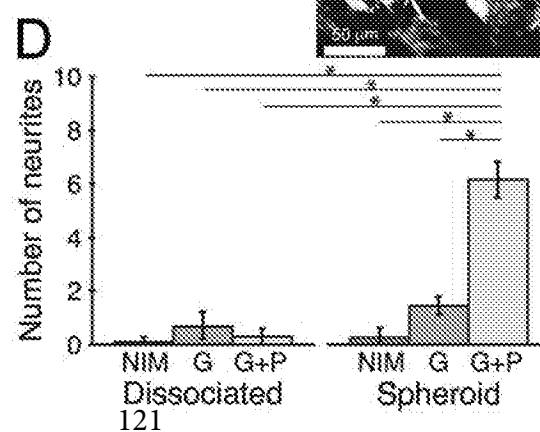
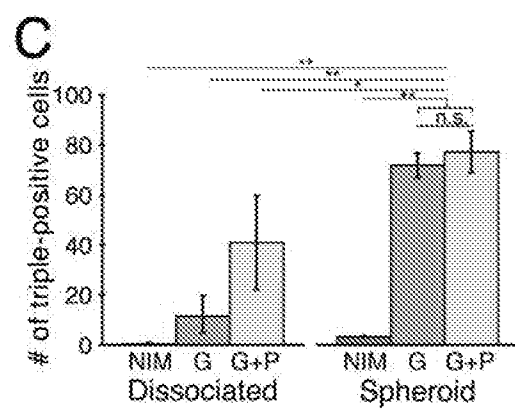
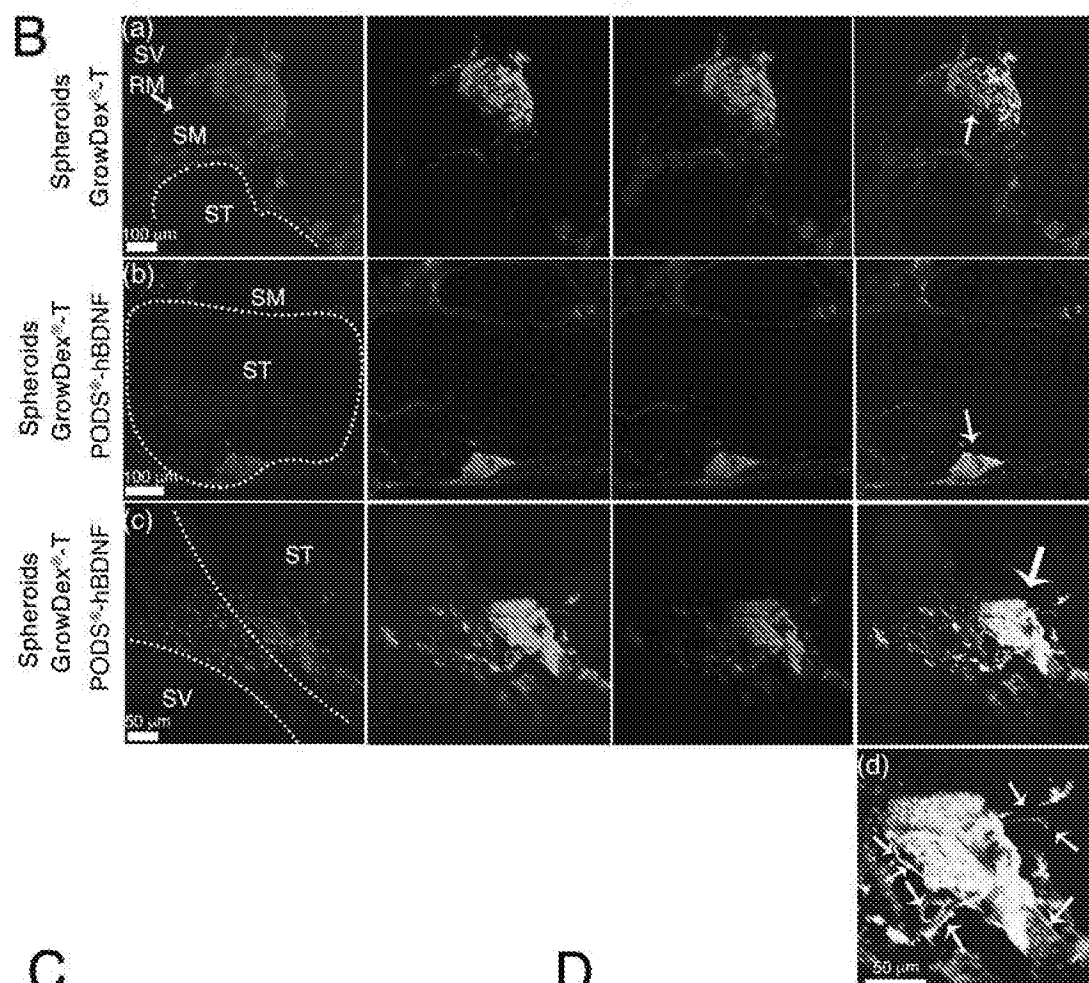
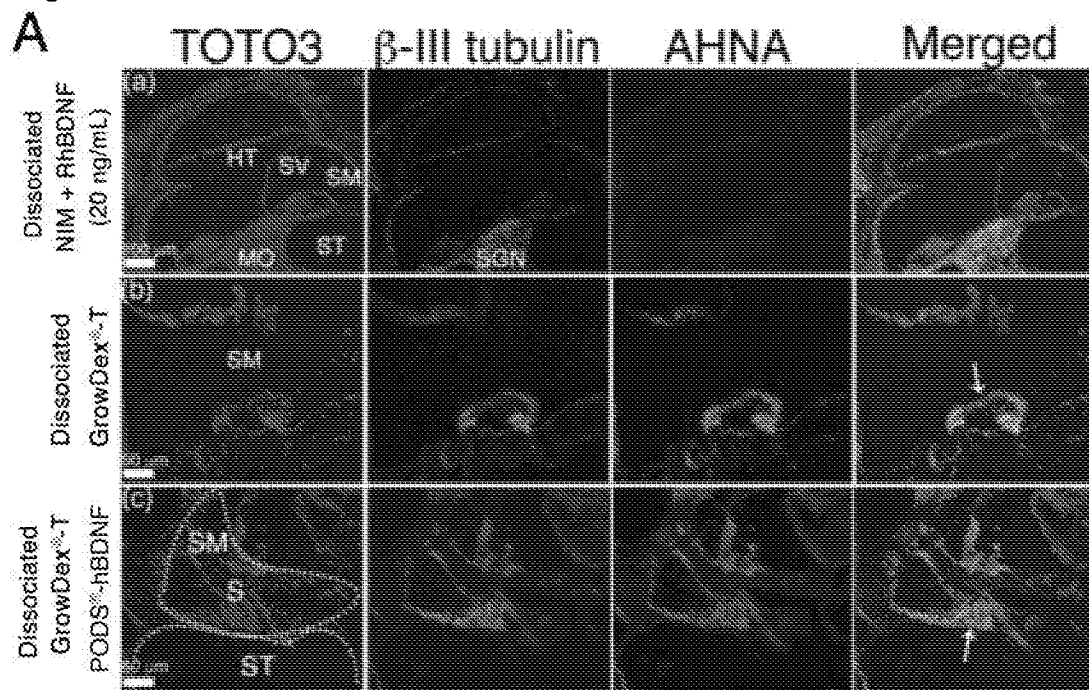
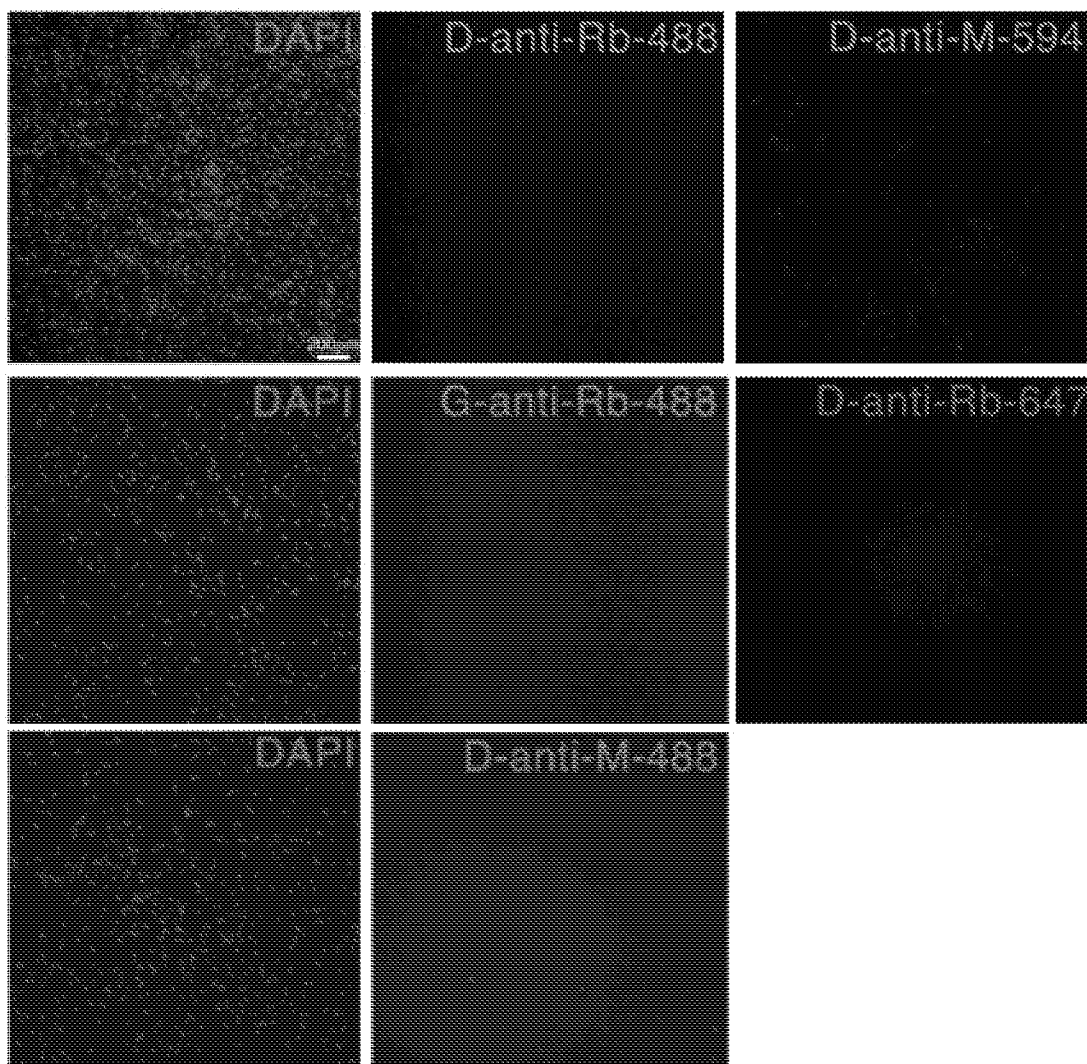
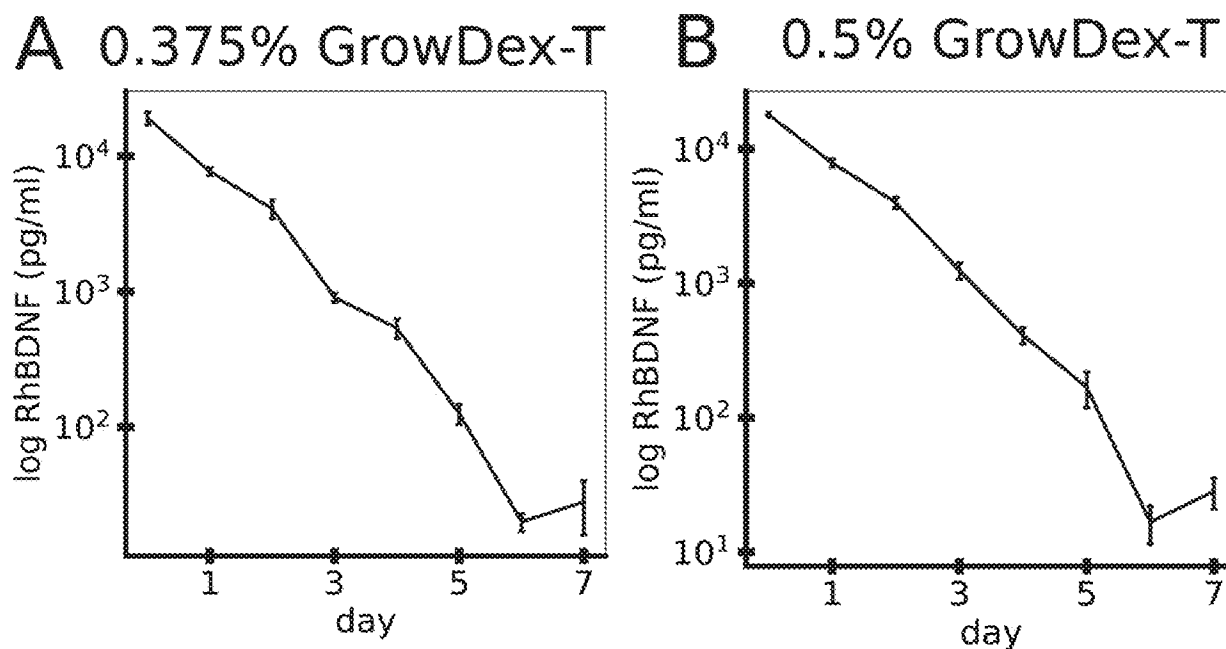


Figure 9

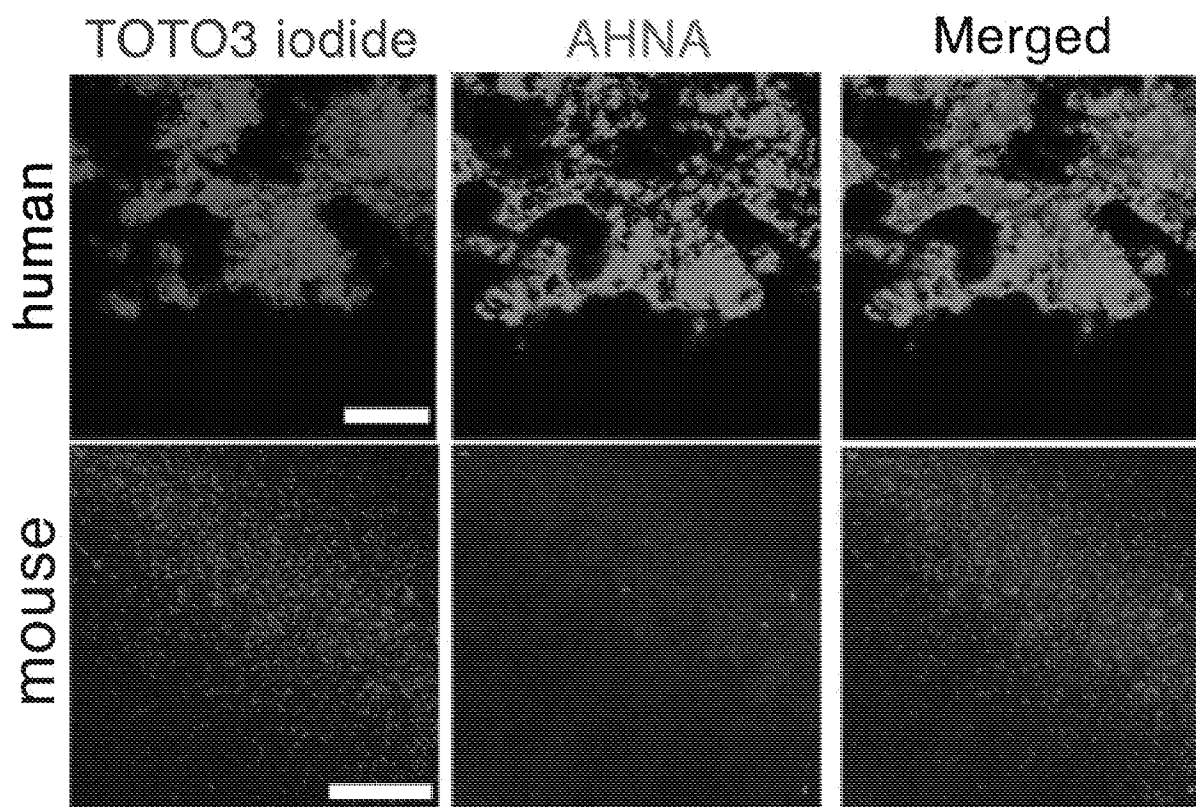




Supplementary Figure S1: Fluorescence images for a secondary antibody control experiment using donkey anti-rabbit Alexa 488 (D-anti-Rb-488), and donkey anti-mouse (D-anti-M-594) **(A)** , goat anti-rat Alexa 488 (G-anti-Rb-488), and donkey anti-mouse (D-anti-Rb-647) **(B)**, donkey anti-mouse Alexa 488 (D-anti-M-488) **(C)**. Each sample was treated identically to experimental immunocytochemistry conditions with the omission of primary antibody during overnight incubation. DAPI image was taken followed by a secondary antibody only condition. D: donkey, G: goat, M: mouse, and Rb: rabbit.



Supplementary Figure S2: Degradation kinetics of 20 ng/mL RhBDNF. **A**: 0.375% GrowDex[®]-T. **B**: 0.5 % GrowDex[®]-T. Note that a base-10 log scale is used for the Y axis.



Supplementary Figure S3: Confocal fluorescent photomicrographs of a positive and negative control sample stained with STEM101, anti-human nuclear antibody (ANHA, red) and TOTO3 iodide counterstain, blue). Top row: human tissue (positive control). Bottom row: mouse brain tissue (negative control). Stains have been pseudo-colored. Scale bar: 250 μm .

Primary Antibody	Application	Catalog #	Manufacturer	Location
Anti-PAX8	1:100	ab13611	Abcam	Cambridge, MA
Anti-SOX2	1:500	ab97952	Abcam	Cambridge, MA
Anti-Nestin	1:100	MAB353	MilliporeSigma	Burlington, MA
Anti- β -III Tubulin	1:100	T7816	MilliporeSigma	Burlington, MA
Anti-Neurogenin1	1:100	ab89461	Abcam	Cambridge, MA
Anti-E-Cadherin	1:250	610181	BD Biosciences	San Jose, CA
Anti-Myosin VIIA/MYO7A	1:250	ab3481	Abcam	Cambridge, MA
Anti-PAX6	1:250	ab195045	Abcam	Cambridge, MA
Anti-MAP2	1:50	sc-74421	Santa Cruz	Dallas, TX
Anti-VGLUT2	1:500	AB2251-I	MilliporeSigma	Burlington, MA
Anti-Peripherin	1: 250	NB300-137	Novus Biologicals	Centennial, CO

Supplementary Table S1: A table of primary antibodies that were used for immunocytochemistry in this study.

Secondary Antibody	Catalog #	Manufacturer	Location
donkey anti-mouse Alexa Fluor 594	R37115	Thermo Fisher Scientific	Waltham, MA
donkey anti-rabbit Alexa Fluor 647	A-31573	Thermo Fisher Scientific	Waltham, MA
donkey anti-sheep Alexa Fluor 488	A-11015	Thermo Fisher Scientific	Waltham, MA
donkey anti-rabbit Alexa Fluor 488	A-21206	Thermo Fisher Scientific	Waltham, MA
donkey anti-mouse Alexa Fluor 488	ab150105	Abcam	Cambridge, MA
donkey anti-rabbit Alexa Fluor 594	ab150076	Abcam	Cambridge, MA
donkey anti-goat Alexa Fluor 488	ab150129	Abcam	Cambridge, MA
goat anti-sheep Alexa Fluor 594	A-11016	Thermo Fisher Scientific	Waltham, MA
goat anti-guinea pig Alexa Fluor 488	A-11073	Thermo Fisher Scientific	Waltham, MA
goat anti-rabbit Alexa Fluor 647	A-27040	Thermo Fisher Scientific	Waltham, MA
donkey anti-mouse Alexa Fluor 647	ab150107	Abcam	Cambridge, MA
goat anti-rat Alexa Fluor 555	A-21434	Thermo Fisher Scientific	Waltham, MA

Supplementary Table S2: A table of secondary antibodies that were used in this study. Note that the concentration of the secondary antibodies was 5 µg/mL except donkey anti-mouse Alexa Fluor 647 (4 µg/mL).