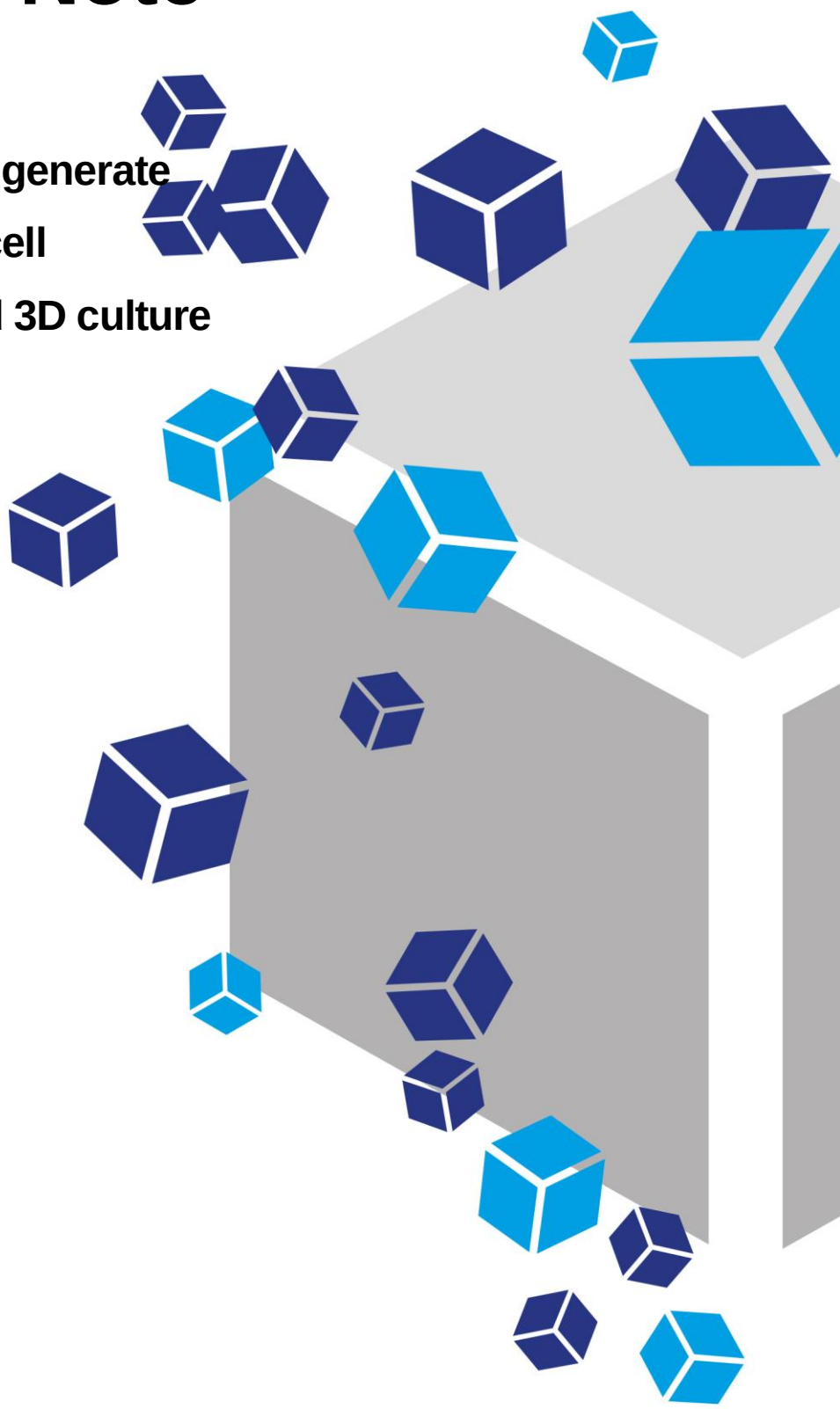
Application Note

PODS® hBDNF crystals generate superior otic neuronal cell differentiation in 2D and 3D culture



PODS® hBDNF crystals generate superior otic neuronal cell differentiation in 2D and 3D culture

Data Courtesy of Dr Akihiro Matsuoka, Northwestern University, Chicago, IL, USA

Introduction to PODS®

The challenge for conventional growth factors

Many proteins, especially growth factors and cytokines, when used as a reagent, degrade quickly, rapidly losing their bioactivity. Additionally, they can also suffer from lot-to-lot product variation. This fragility and variability hampers research and significantly limits the therapeutic potential of proteins.

Protein Micro-depots

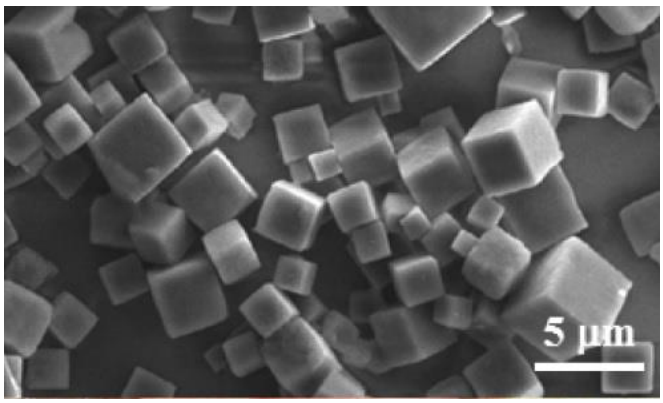
Development of a technology that can continuously replenish active protein from a local, microscopic store, has been a significant challenge, but one that could transform the fields of cell culture and medicine by allowing greater control and reproducibility of cell culture.

Introducing PODS®

PODS® is a sustained release system which continuously replenishes proteins from millions of local microscopic stores which can be placed next to (or at a distance from) cells, either randomly or in precise locations. Just like cells, these micro-depots release a steady stream of bioactive protein. This protein can be limited to local surroundings or dispersed more widely, or made to form a gradient.

How does it work?

At the heart of PODS® is an extraordinary polyhedrin protein. This specific polyhedrin protein has the unique ability to encase cargo proteins within perfect, transparent, cubic, micro-sized crystals, much smaller than the cells. These protein crystals form admixtures of the polyhedrin and cargo proteins which slowly degrade releasing the biologically active cargo protein.



How can PODS® help my research?

PODS® are tough and will withstand physical and chemical stress, so you can handle them with ease. PODS® typically release intact cargo protein over several weeks and months. Using PODS® you can readily create a steady-state protein environment in microscopic detail wherever you want, tailored exactly to your requirements. This is the power of PODS®. PODS® proteins are now available for many growth factors and cytokines and are already being used in many leading world-class research labs. PODS® protein applications include:

- Micropatterning
- Physiological, stable gradient formation
- Bioinks for 3D printing
- Microcarriers
- Functionalizing scaffolds
- Microfluidics (lab on a chip)
- Improved and simplified stem cell culture
- Therapeutic protein delivery

Overview

Inner ear regeneration utilizing human embryonic stem cell (hESC)-derived Otic Neuronal Progenitors (ONPs) has remarkable potential for treating sensorineural hearing loss. The local environment of the inner ear requires a suitable stem cell niche to allow hESC-derived ONP engraftment as well as neuronal maturation.

In vitro culture systems can be utilized to study and model a suitable stem cell niche to promote neuronal differentiation. To enhance late stage *in vitro* neuronal differentiation of hESC-derived ONP spheroids, PODS® human brain neurotrophic growth factor (hBDNF) crystals, providing a sustained release delivery system, were used in combination with a nanofibrillar cellulose hydrogel (GrowDex®-T).

These observations demonstrate that sustained release from a single dose of PODS® hBDNF enabled highly effective Late ONP differentiation into otic neuronal lineages, both in 2D and 3D *in vitro* culture. In comparison, conventional BDNF growth factor treatment had limited efficacy.

Methods

Directed differentiation of hESCs into otic neuronal cell lineages

For a detailed protocol, please refer to the original manuscript[1]. Briefly, human embryonic stem cell lines (hESC) lines H1, H7 and H9 were differentiated into ONPs using a directed neuronal differentiation protocol (Figure 1). A stepwise series of ligands and growth factors was added to promote hESC differentiation through the early stages and towards the Late ONP lineage. At day 25, hESC-derived ONPs were subsequently differentiated further into otic neuronal cell lineages in both 2D and 3D culture formats.

For 2D monolayer differentiation, Late ONPs (day 25) were treated with either PODS® hBDNF ($8x10^5$) or conventional BDNF growth factor (20 ng/ml) for 7 days in neuronal induction medium (NIM). No media changes were performed throughout the 7-day period. For 3D differentiation, hESC-derived Late ONPs (day 25) were dissociated and cells seeded into EZSPHERE® plates and cultured at 37°C for 2 days to generate ONP spheroids. Thereafter, spheroids were injected into nanofibrillar cellulose hydrogels (GrowDex®-T; UPM Biomedicals, catalogue number: 200103002) that were pre-mixed with either PODS® hBDNF crystals ($8x10^5$) (catalogue number PPH1) or conventional hBDNF growth factor (20 ng/ml). Embedded ONP spheroids were cultured *in vitro* for a further 14 days in NIM. A full media change was performed after 7 days.

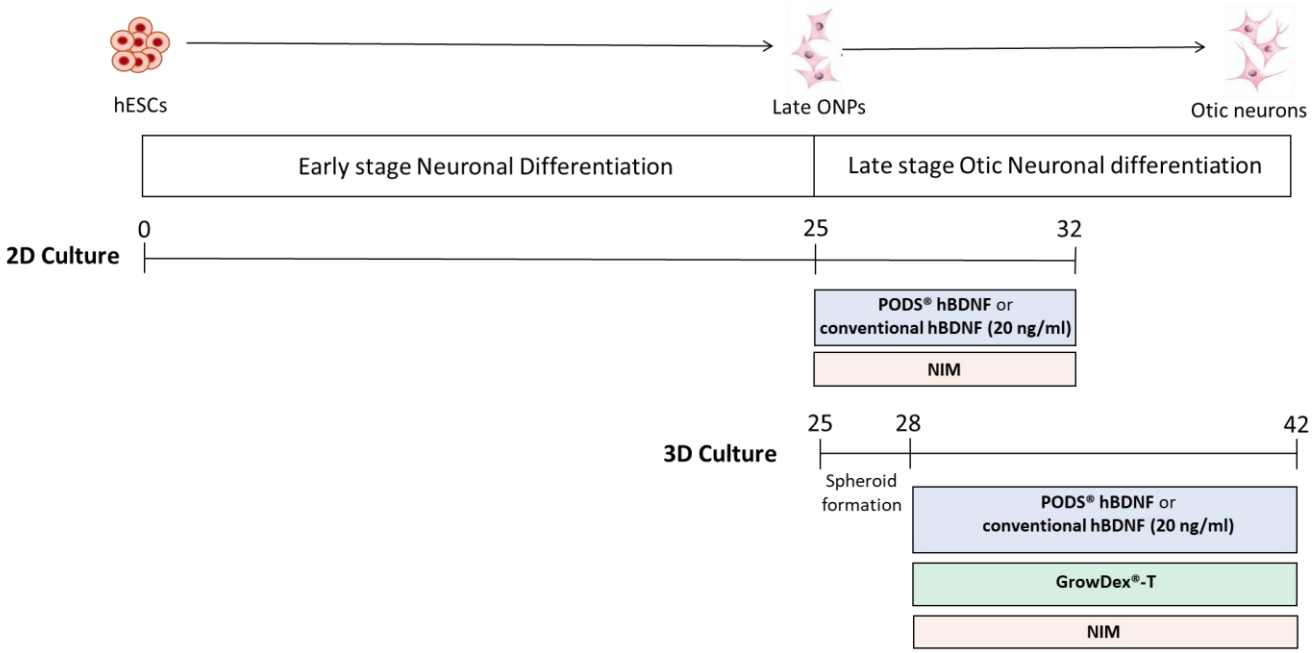


Figure 1. Directed neuronal differentiation protocol for hESC-derived otic neuronal cell lineages in 2D and 3D *in vitro* culture formats. Schematic summary outlining the differentiation of pluripotent hESCs into otic neuronal cell lineages. For 2D neuronal differentiation, PODS® hBDNF crystals or conventional hBDNF growth factor treatment was initiated on day 25. Cells were cultured in NIM for 7 days. For 3D otic neuronal differentiation, hESC-derived Late ONPs (day 25) were dissociated and used to generate ONP spheroids. These were then transferred to culture plates containing GrowDex®-T pre-mixed with either PODS® hBDNF crystals or conventional human BDNF growth factor. Embedded ONP spheroids were cultured in NIM for a further 14 days. Figure adapted from (1).

Results

PODS® hBDNF supports Late otic neuronal differentiation in 2D monolayer culture

In order to demonstrate the efficacy and efficiency of the sustained-release of PODS® hBDNF, PODS® were first utilized in a 2D monolayer culture of Late ONPs, without the nanofibrillar cellulose hydrogel GrowDex®-T (Figure 1). Compared to Late ONPs treated with conventional hBDNF growth factor (20 ng/ml), the PODS® hBDNF-treated cells expressed high levels of specific neuronal markers: GATA3, Neurogenin-1 and PAX8 (otic lineage markers); SOX2 and nestin (neuronal progenitor markers); vesicular glutamate transporter-2 (glutamatergic neuronal marker); β-III tubulin (pan-neuronal marker) and peripherin (peripheral sensory neuron marker) (Figure 2A). Quantification of these markers shows that the proportion of nestin- (purple) and SOX2- (green) positive cells were expressed at significantly lower levels than other markers, suggesting that the treatment facilitated otic neuronal differentiation.

When further analysing nestin- and SOX2-positive cells, PODS® hBDNF treatment significantly decreased expression levels of nestin (~30% reduction) and SOX2 (~20% reduction) compared to conventional BDNF growth factor treatment (Figure 2B), suggesting that PODS® treatment more effectively differentiated Late ONPs into otic neuronal lineages.

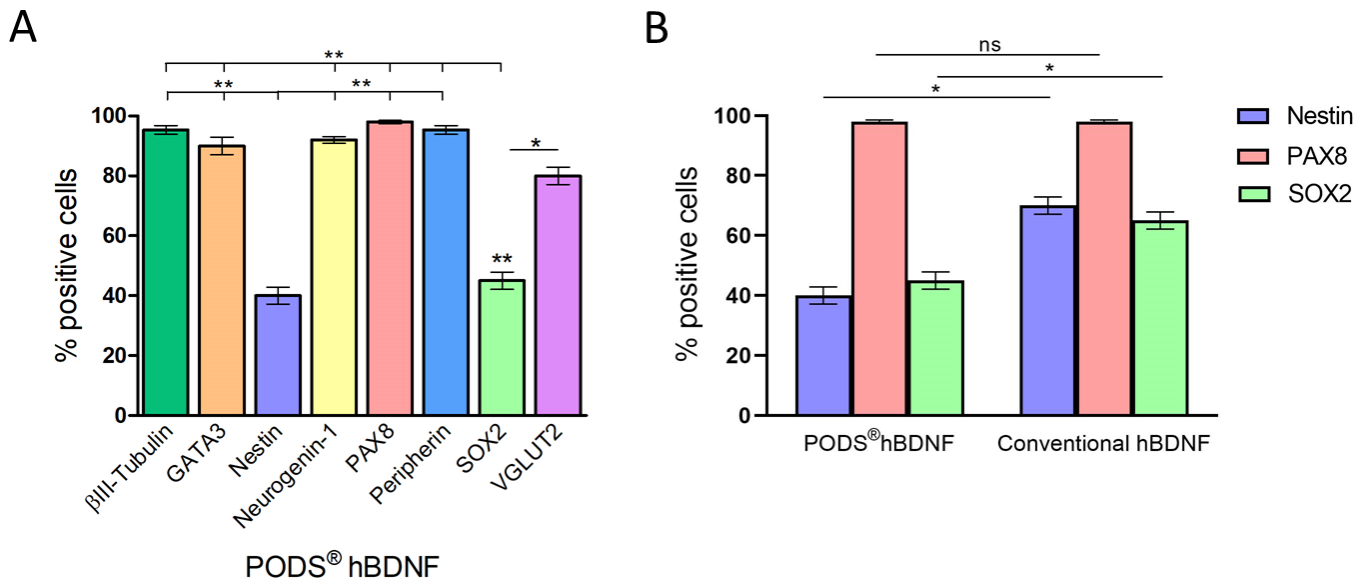


Figure 2. PODS® hBDNF supports the induction of Late otic neuronal differentiation in 2D culture (A) Quantification of the otic neuronal markers (% positive staining, *n*=3) on hESC-derived Late ONPs treated with PODS® hBDNF for 7 days. (B) Quantification of % positive staining for nestin, PAX8, and SOX2 on cells treated for 7 days with either PODS® hBDNF or with conventional hBDNF. $*p < 0.05$, $**p < 0.01$ by one-way ANOVA with Tukey's *post-hoc* test. n.s.: not statistically significant. Figure adapted from [1].

PODS® hBDNF supports Late otic neuronal differentiation in 3D spheroid culture

Having demonstrated efficacy in 2D culture, PODS® hBDNF crystals were then utilized to promote Late otic neuronal differentiation in a 3D culture format. Late stage ONP spheroids were generated as described above and then cultured for a further 14 days in either i) NIM only; ii) NIM with GrowDex®-T hydrogel (to mimic a stem cell niche); iii) NIM with PODS® hBDNF embedded in GrowDex®-T; or iv) NIM with conventional hBDNF mixed into GrowDex®-T.

PAX8 expression peaks at the otic placode or otocyst stages (corresponding to the ONP stage) and is thereafter downregulated. Immunocytochemistry of hESC-derived ONP spheroids cultured with PODS® hBDNF for 7 days (Figure 3, upper panel) show both significantly enhanced neurite outgrowth (β-III tubulin; red) as well as reduced expression levels of PAX8 (green), when compared to conventional hBDNF (Figure 3, lower panel).

Quantification of PAX8 expression via immunohistochemistry was used to gauge the degree of otic neuronal differentiation. The low levels of PAX8 expression observed in hESC-derived ONP spheroids cultured in PODS® hBDNF (Figure 4A, red) compared to all other conditions indicate PODS® hBDNF treatment promotes neuronal differentiation most efficiently. Moreover, based on PAX8 expression levels, the PODS® hBDNF/GrowDex®-T 3D culture proved to be more highly differentiated than the earlier 2D-monolayer ONP culture with PODS® BDNF (Figure 2A-B).

Furthermore, the quantification of neurite length, neurite bearing cells and neurite arborization (Figure 4B-D) demonstrated that axonal branching was significantly greater in culture conditions with PODS® hBDNF/GrowDex®-T (red), in contrast to conventional BDNF growth factor or culture conditions without hBDNF.

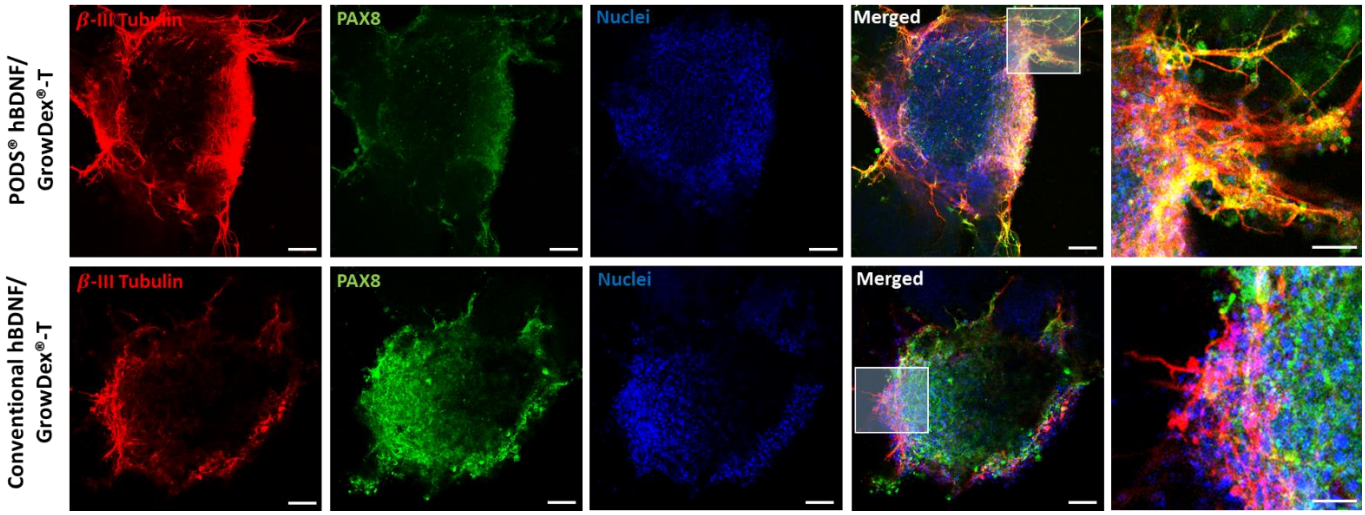


Figure 3: PODS® hBDNF supports Late otic neuronal differentiation of hESC-derived ONP spheroids cultured with 0.375% GrowDex®-T. Immunocytochemistry of neuronal markers (β-III Tubulin, PAX8) in hESC-derived ONP spheroids cultured with PODS® hBDNF or conventional hBDNF for 7 days. Scale bars: 100 µm.

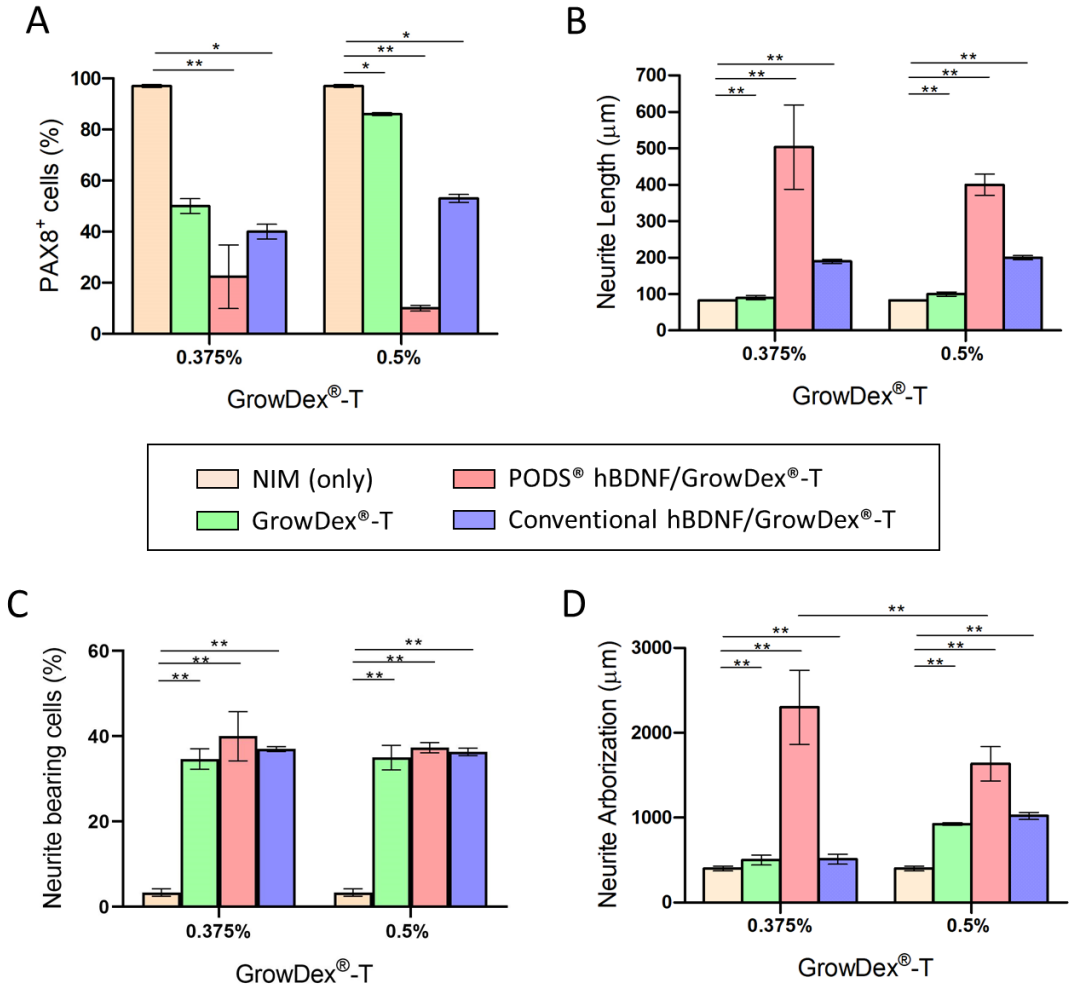


Figure 4: PODS® hBDNF supports Late otic neuronal differentiation of hESC-derived ONP spheroids cultured with GrowDex®-T. Quantification of (A) PAX8-positive expression based on immunocytochemistry in hESC-derived ONP spheroids cultured in: NIM (only); GrowDex®-T; PODS® hBDNF/GrowDex®-T or conventional hBDNF/GrowDex®-T. Quantification of (B) neurite length; (C) neurite bearing cells and (D) neurite arborization, arising from hESC-derived ONP spheroids cultured in 0.375% and 0.5% GrowDex®-T hydrogels (n=3). $*p<0.05$, $**p<0.01$, $***p<0.001$ by 1-way ANOVA with Tukey's *post-hoc* test. Figure adapted from (1).

Conclusions

- PODS® hBDNF treatment differentiates Late hESC-derived ONPs into otic neuronal lineages consistently more efficiently compared to conventional BDNF growth factor, in both 2D and 3D *in vitro* culture formats.
- PODS® hBDNF crystals can be readily incorporated into nanofibrillar cellulose hydrogels such as GrowDex® delivering bioactive growth factors in a sustained manner.
- PODS® hBDNF treatment in hydrogels dramatically improves further neuronal differentiation in Late ONP spheroids compared to conventional BDNF growth factor, as characterized by greater neurite length and neurite axonal branching and reduced PAX8 expression.
- For longer term culture periods (≥7 days), a single dose of PODS® hBDNF is highly effective in facilitating otic neuronal differentiation in both 2D and 3D culture, improving growth factor dosing levels, reducing hands-on time, experimental complexity and the cost of materials.

Reference

1) 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 Biomaterials. 2020 May;108:111-127.

For more information and a full list of our current PODS® growth factors, please visit our website www.cellgs.com.

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