STEMdiff™ Cerebral Organoid Kit: A New Tool for the Culture of 3-D Brain Organoids Derived from Human Pluripotent Stem Cells

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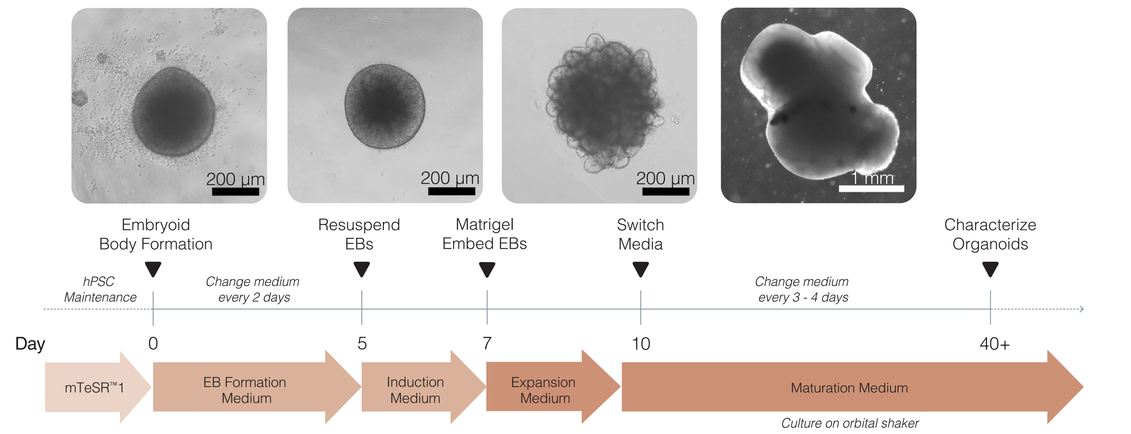
Introduction

2-D neural cultures derived from human pluripotent stem cells (hPSCs), including human embryonic and induced pluripotent stem cells (hESCs or hiPSCs), are useful models with which to study the nervous system, but they are limited in their capacity to fully recapitulate the complex organization of brain tissues. Lancaster et al.[1,2] established a hPSC-based organoid culture system that models the major features of early human brain development. Based on the published media formulations, we developed the STEMdiff™ Cerebral Organoid Kit to enable generation of organoids in a simple and highly reproducible manner. This kit contains 2 basal media and 5 supplements, which are combined to prepare four separate complete media corresponding to the 4 stages of cerebral organoid formation. hPSCs maintained in mTeSR1™ were single-cell dissociated and cultured in Embryoid Body (EB) Formation Medium in U-Bottom plates (day 1 - 5, Stage 1). The resulting EBs were then transferred to Induction Medium (day 6 - 7, Stage 2); next, they were expanded by embedding in Corning® Matrigel® and cultured in Expansion Medium (day 7 - 10, Stage 3). The expanded organoids were then cultured in Maturation Medium, with agitation, for extended periods of time (day 10 - 40+, Stage 4). Morphological analysis of organoids was performed on days 5, 7, 10 and 40, which are the endpoints of Stages 1 - 4 respectively. Organoids at Day 40 were analyzed by RT-qPCR or cryosectioned and processed for immunofluorescence (>3 organoids per analysis; 2 hESCs, n = 2 and 2 iPSCs, n = 2). We achieved high efficiencies across multiple cell lines (2 hESCs, n = 2 and 2 iPSCs, n = 2) for EB generation (100% success, n = 128/128), expansion (>95% exhibited extensive folding of neuroepithelia, n = 104/107) and maturation (>60% of organoids were >1 mm in diameter with dense cores, n = 62/94). These outcomes are a significant improvement upon the published protocol and reagents, with which <30% of the generated organoids had the desired morphology. In vivo, the human cortex consists of progenitor and neuronal populations that organize into distinct layers. The mature organoids generated here exhibited a similar architecture with neural progenitors (SOX2+, PAX6+) localized in apical regions surrounding a central ventricle. Adjacent to the apical progenitors, neuronal progenitors (TBR2+, Ki-67+) were found abutting neurons (CTIP2+, MAP2+, TBR1+), resembling the intermediate zone and cortical plate regions. Taken together, our data demonstrate that the STEMdiff™ Cerebral Organoid Kit supports highly efficient generation and expansion of cerebral organoids, and improves upon currently available methods.

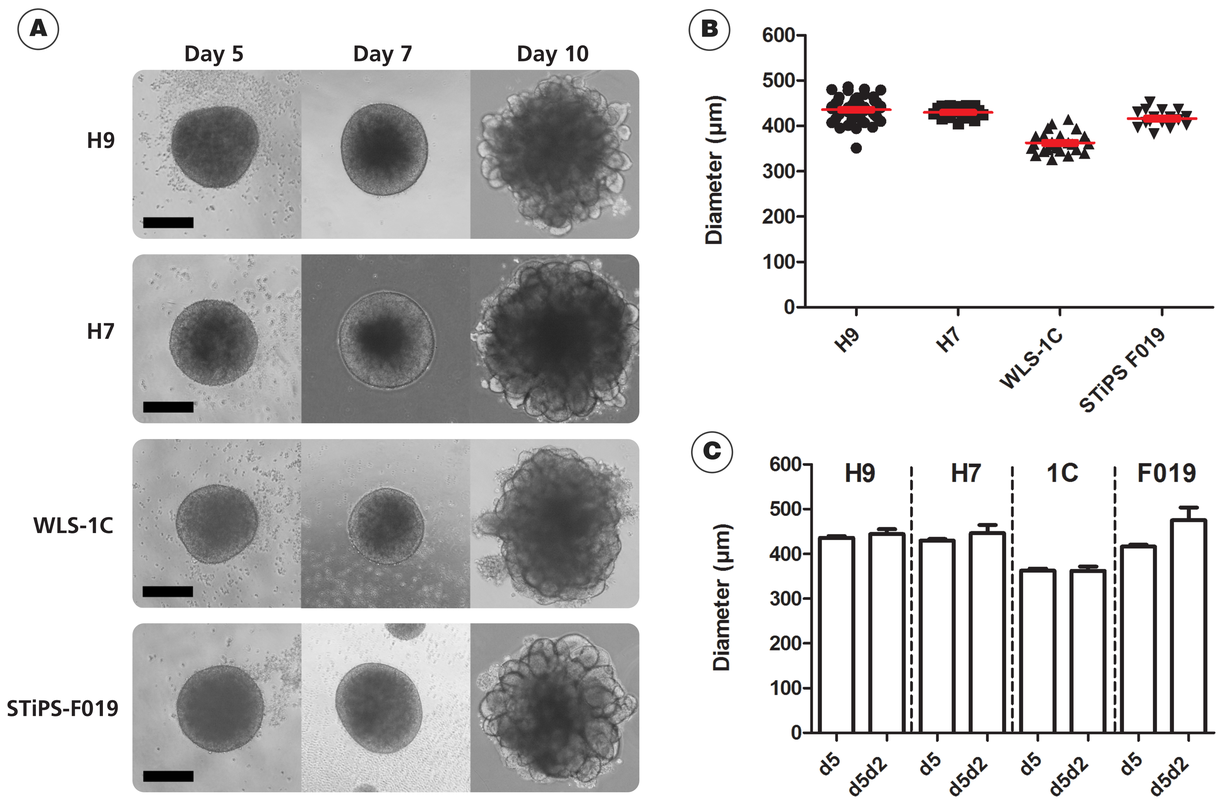
Methods



Human pluripotent stem cells (either embryonic or induced pluripotent stem cells) maintained in mTeSR1™ were dissociated into single-cell suspensions using Gentle Cell Dissociation Reagent (GCDR) and seeded at a density of 9,000 cells/well in a U-Bottom 96-well Ultra Low Attachment Plate (Corning) in EB Formation Medium + 10 _M Rho-Kinase Inhibitor (ROCKi). EBs were fed every 2 days with EB Formation Medium without ROCKi. After 5 days, EB diameters were measured from images taken using the Incucyte system (Essen BioScience) and they were transferred to Induction Medium in a 24-well Ultra low Attachment Plate (Corning®). EBs were cultured for an additional 2 days and were then embedded in liquid Matrigel® (Growth Factor Reduced, Corning®) followed by transfer to a non-tissue culture treated 6-well plate (12 -16 organoids/well). Embedded organoids were maintained in Expansion Medium for 3 days. On Day 10, organoids were switched to Maturation Medium and cultured on an orbital shaker set at 57-95 RPM (Infors HT). Organoids were fed every 3 – 4 days with Maturation Medium. On Day 40, organoids were analyzed for neuronal (TBR1, CTIP2, MAP2, beta III Tubulin), intermediate progenitor (ASCL1, TRB2) and neural progenitor (PAX6, SOX2) markers using either RT-qPCR or immunostaining following cryosectioning.

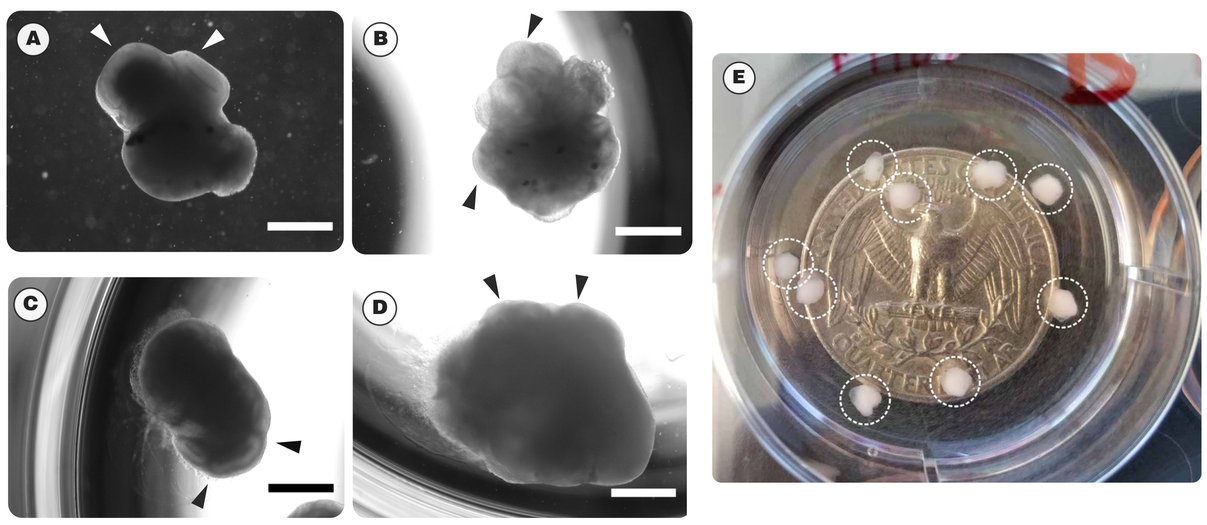
Results

Figure 1. The STEMdiff™ Cerebral Organoid Kit Supports EB Formation and Neuroepithelia Expansion for Multiple hPSC Lines



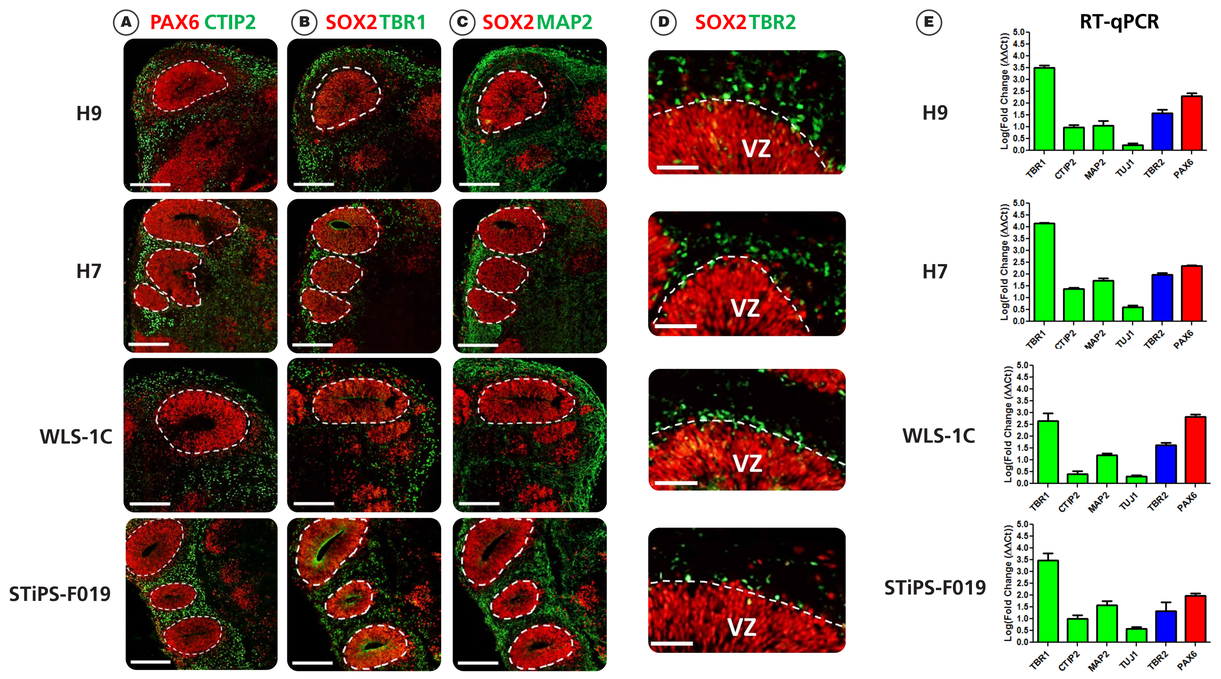
(A) Robust early organoid formation was observed in four hPSC lines (2 hESC; H9 and H7, 2 hiPSC; WLS-1C and STiPS-F019) (scale bar = 300 µm). **Day 5:** Embryoid Body Formation; Single-cell suspensions of hPSCs formed spherical aggregates with smooth edges. **Day 7:** Induction stage; EB edges smoothened and developed a translucent quality. **Day 10:** Expansion stage; Matrigel® embedded EBs displayed expanded neuroepithelia evident by bubbling of their surface. Over 80% of all embedded organoids exhibit this type of morphology indicative of neuroepithelia expansion (n = 2 per hPSC line, 8 to 24 organoids measured per hPSC line). **(B)** Diameter of EBs measured at day 5 shows that all EBs grew to a diameter over 300 µm (n = 2 per cell line, 16 – 48 EBs measured per hPSC line). **(C)** Measurement of EBs taken at Day 7 (d5d2) show that EBs maintain their size when switched to Induction Medium (n = 2 per cell line. 16 – 48 organoids measured per hPSC line).

Figure 2. Day 40 Cerebral Organoids Grow to Over 1 mm in Diameter and Exhibit Tissue Structures with Dense Cores



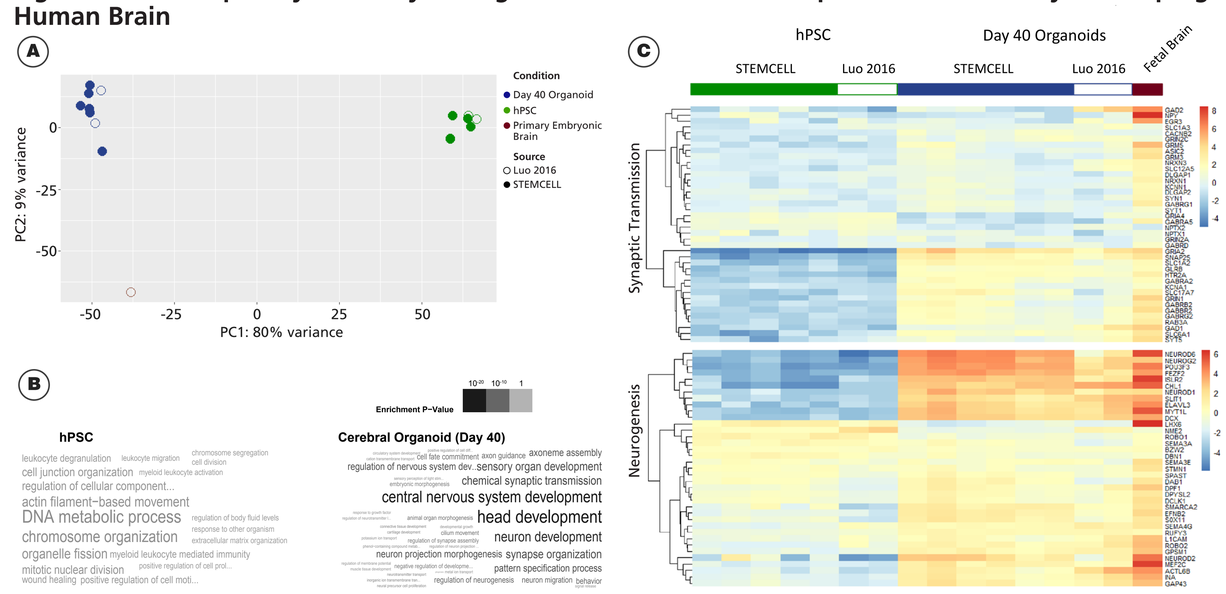
Cerebral organoids cultured in Maturation Medium at day 40 developed dense tissue-like structures and areas that exhibit layering (arrowheads). More than 60% of organoids from each experiment exhibited this type of morphology (n = 2 per cell line, 8 – 24 Organoids measured per hPSC line) **(A)** H9 **(B)** H7 **(C)** WLS-1C **(D)** STiPS-F019. **(E)** Size of day 60 organoids (H9). An American quarter is used for scale. Cerebral organoids are outlined (white dotted circles).

Figure 3. Day 40 Cerebral Organoids Contain Progenitor and Neuron Populations that Organize into Distinct Layers



Immunostaining was performed on 12 µm cryosections of 4% paraformaldehyde fixed organoids for the following markers: **(A)** PAX6/CTIP2, **(B)** SOX2/TBR1, **(C)** SOX2/MAP2, **(D)** SOX2/TBR2 (scale bar = 200 µm). Neural progenitors (SOX2+, PAX6+) localized in distinct apical regions surrounding a central ventricle. **(E)** RT-qPCR analysis showed upregulation of both neural progenitor and mature neuron transcripts as Log(Fold Change ΔΔCt) (Average ± SEM n = 2 per cell line, ≥ 3 organoids per analysis). Data is normalized to 18S/TBP and compared to undifferentiated hPSC control.

Figure 4. RNA-Seq Analysis of Day 40 Organoids Reveals Marker Expression of the Early Developing Human Brain



(A) Principal component analysis of hPSC and cerebral organoid transcriptomes. Cerebral organoids generated using the STEMdiff™ Cerebral Organoid Kit (filled blue circles) cluster together, and cluster with previously published[3] cerebral organoids (open blue circles). The first principal component accounts for the majority of variance seen (PC1; 80%) and distinguished the cerebral organoid samples from the hPSCs (green circles). The second principal component accounts for only 9 % of the variation, and highlights the modest gene expression differences between cultured organoids and primary embryonic fetal brain samples (19 post-conceptional weeks, brown circles). **(B)** Gene ontology terms enriched in Day 40 cerebral organoids with respect to hPSCs reveals transcripts related to brain development and function. **(C)** Heatmap of expression levels for genes associated with synaptic transmission function and neurogenesis in Day 40 organoids. These data show that gene expression of cerebral organoids generated from the STEMdiff™ Cerebral Organoid Kit are similar to published results.

Summary

STEMdiff™ Cerebral Organoid Kit

- Supports highly efficient generation of hPSC-derived cerebral organoids from multiple hPSC lines
- Generates organoids with cortical architecture consisting of both neural progenitor and mature neuron populations
- Reproduces cerebral organoids that are consistent with published results by Lancaster et al.[1,2]

References

1. Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature, 501(7467), 373–9.
2. Lancaster, M.A, & Knoblich, J.A. (2014). Generation of cerebral organoids from human pluripotent stem cells. Nature Protocols, 9(10), 2329–2340.
3. Luo, C., Lancaster, M.A., Castanon, R., Nery, J.R., Knoblich, J.A., Ecker, J.R. (2016) Cerebral Organoids Recapitulate Epigenomic Signatures of the Human Fetal Brain. Cell Reports, 17(12), 3369-3384

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