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Research article

Six-month cultured cerebral organoids from human ES cells contain matured neural cells

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ABSTRACT

Recently, researchers have developed protocols for human cerebral organoids using human pluripotent stem cells, which mimic the structure of the developing human brain. Existing research demonstrated that human cerebral organoids which undergo short cultivation periods, contain astrocytes, neurons, and neural stem cells, but lacked mature oligodendrocytes, and mature, fully functional neurons. In this study, we analyzed organoids induced from H9 human embryonic stem (ES) cells that were cultivated for as long as six months. We observed mature oligodendrocytes, positive for MBP (myelin-basic protein), and mature GAD67 (glutamate decarboxylase 67 kDa isoform)-positive inhibitory neurons and VGLUT1 (vesicular glutamate transporter 1)-positive excitatory neurons via immunohistochemical analysis. These observations suggest that long-term cultivation of cerebral organoids can lead to the maturation of human cerebral organoids, which can be used as a tool to study the development of human brains.

1. Introduction

Pluripotent stem cells, such as embryonic stem (ES) cells (ESCs) [1–3] and induced pluripotent stem cells [4–6], are known to give rise to almost all the cells found in the three germ layers, and a number of research groups have since published protocols on the induction of various cell types from human ES cells using 2D cultures, based on fine-tuned growth factors. The inability to replicate the growth of organs in a 3D environment based on such 2D cultures is a limitation that has prompted recent attempts in the development of tissue organoids that mimic the multicellular structure of selected organs, such as the human brain [7–11], colon [12], kidney [13], and retina [14,15].

In particular, the mammalian cerebral cortex is developed in a 3D environment during the embryonic period and composed of many layers of neuronal cells, growing with well-tuned interactions mediated by cell–cell signaling or the secretion of various growth factors. Its complex development makes it difficult to analyze these structures by 2D in vitro culture methods. As is known in the field of human brain research, the human brain has a more complex structure than that of a mouse, thus making it challenging to recapitulate the rapid expansion of the cortex in the developing human brain using mouse models [16,17]. Therefore, 3D-cultured human cerebral organoids are expected to become a powerful tool to analyze the complex developing human brain without the associated ethical concerns in acquiring human tissue samples [18].

Currently, human cerebral organoids have been demonstrated to recapitulate some of the earliest stages of human embryonic brain development [19]. Additionally, neuronal populations show maturation during extended cultivation. One group revealed that six-month-old cerebral organoids contain mature astrocytes and mature neurons, such as putative callosal, and putative corticofugal neurons, via single-cell RNA sequencing [20]. They also detected neurons with synapses, and spontaneously active neuronal networks in cerebral organoids that were cultivated for as long as eight months [20]. A separate research group reported the maturation of cerebral organoids after about eight months of cultivation [21]. They observed oligodendrocyte progenitor

Abbreviations: EB, embryoid body; ESC, embryonic stem cell; GAD67, glutamate decarboxylase 67 kDa isoform; GFAP, glial fibrillary acidic protein; MBP, myelin-basic protein; OLIG1/2, oligodendrocyte transcription factor 1/2; SOX2/10, SRY-box 2/10; S100β, S100 calcium binding protein B; VGLUT1, vesicular glutamate transporter 1

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cells (OPC) expressing OPC markers, such as O4 (O antigen 4) and OLIG1 (oligodendrocyte transcription factor 1), by immunohistochemistry. It is of note, however, that no group has reported mature oligodendrocytes with MBP expression. In the present study, we intended to induce maturation of human cerebral organoids after an extended six-month cultivation. We expect that human cerebral organoids will enable us to analyze the mechanism of many diseases starting from the later phases of prenatal brain development [22], as well as demyelinating brain injuries/diseases [23,24]. In addition, we expect that the normal process of human brain development and maturation can be analyzed by long-term culture of human cerebral organoids [21].

In this study, we examined the maturation of human cerebral organoids after an extended period of culture. Our findings highlight the novel possibility of human cerebral organoids for research of human brain development, leukodystrophies, hereditary epilepsies, and various demyelinating diseases.

2. Materials and methods

2.1. Maintenance of ES cells

Human H9 ESC line (46XX, WA09, WiCell Research Institute, Madison, WI, USA [1]) was purchased from WiCell and maintained in mTeSRTM1 medium (05851, Stemcell Technologies) based on feeder-free culture protocols on six-well plates (3506, Corning) coated with growth factor-reduced Matrigel (356230, BD Biosciences) with Rho Kinase (ROCK) inhibitor (final concentration 10 μM, S-1049, Selleck Chemicals). The cells were maintained with daily medium change without ROCK inhibitor until they reached about 70% confluency. They were then detached using versene solution (15040-066, Thermo Fisher Scientific) and seeded with a ratio of 1:20.

2.2. Cerebral organoid development

Human ES cells were detached and subjected to embryoid body (EB) induction based on a published protocol with a slight modifications [11,22]. ESCs were briefly detached using versene and accutase (A6964, Sigma), dissociated into single cells and suspended at 60 cells/μl in 150 μl of the human EB medium (DMEM/F12, 3% FBS, 20% knockout serum replacement, 1 × non-essential amino acids, 1 × Glutamax, and 2-mercaptoethanol) in 96-well clear round bottom ultra-low attachment microplates (7007, Corning). The medium was supplemented with ROCK inhibitor (final concentration 50 μM) and basic fibroblast growth factor (bFGF) (final concentration 4 ng/ml). After six days, each EBs was transferred to an individual well in a 24-well ultra-low attachment plate (3473, Corning), filled with 500 μl of neural induction medium (NIM) per well, composed of DMEM-F12 with 1 × N2 supplement (17502048, Thermo Fisher Scientific), 1 × Glutamax, 1 × non-essential amino acids and heparin (1 μg/ml), and embedded in Matrigel after 5 days. The organoids were induced in organoid medium, described in previously published papers [11,22], on an orbit shaker. The organoid size was analyzed by measuring the area using ImageJ software.

2.3. Immunohistochemical analysis

Each human cerebral organoids was fixed in 4% paraformaldehyde in Phosphate-Buffered Saline (PBS) overnight at 4 °C, dehydrated with 30% sucrose in PBS and embedded in OCT Compound (23-730-571, Thermo Fisher Scientific). Cryostat sections (14 μm) were cut and mounted onto slides (Thermo Fisher Scientific). Mounted sections were incubated for 1 h at room temperature with blocking solution [3% normal donkey serum + 0.3% Triton X-100 in Tris-Buffered Saline (TBS)] and incubated with primary antibodies diluted in blocking solution overnight at 4 °C. After three washes with TBS, corresponding fluorophore-conjugated secondary antibodies diluted in the blocking solution were added and incubated for 2 h at room temperature and followed by DAPI staining. Finally, stained slides were rinsed with TBS three times, mounted and analyzed using a Nikon A1R confocal microscope equipped with four laser lines (405, 488, 561 and 633 nm) under 20× and 40× objective lenses. Serial Z-stack images were obtained and collapsed to obtain a maximum intensity projection of lines. Antibodies specific for TUJ1 (1:400, T8660) and S100β (1:200, S2532) purchased from Sigma, GFAP (1:1000, Z0334) from DAKO, DCX (1:500, SC8066), SOX10 (1:500, SC17343) and SOX2 (1:500, Y17) from SantaCruz, MBP (1:500, AB980) and OLIG2 (1:500, AB9610) and GAD67 (1:500, AB5992) from Millipore, VGLUT1 (1:500, 135-303) from synaptic systems, and AC3 (1:400, D175) from Cell Signaling were used for immunostaining.

2.4. RNA isolation, RT-PCR and quantitative PCR

RNA from human cerebral organoids and ES cells was extracted according to the protocol supplied with TRIzol reagent (15596018, Thermo Fisher Scientific). The concentration and purity of the RNA samples were measured by using DU730 UV–vis Spectrophotometer (Beckman Coulter). The extracted RNA (1 μg) was reverse transcribed according to the protocol supplied with ReverTra Ace qPCR RT Master Mix (FSQ-201, TOYOBO). StepOne Plus Real-time PCR System (Thermo Fisher Scientific) was used to amplify and quantify levels of target gene cDNA. Quantitative real-time PCR (qRT-PCR) was performed with SsoAdvanced Universal SYBR Green Supermix (172-5271, Bio-Rad Laboratories) and specific primers for qRT-PCR. Reactions were run in triplicate and the expression of each gene was normalized to the geometric mean of β-actin as a housekeeping gene and analyzed using the ΔΔCT method. Positive control was human brain cDNA in Human MTC Panel I (636742, TaKaRa), pooled from eight male Caucasians [ages: 43–65 years old]. Primers used in the study are;

GAD67 (XM_017003756.1) [25]

[F: 5′-CCTGGAACTGGCTGAATACC-3′; R: 5′-CCCTGAGGCTTTGTG GAATA-3′],

MBP (NM_002385.2) [26]

F: 5′-GTAGTAAGCCACTCCTTGACTG-3′; R: 5′-GCAGAGAGGACTG TTGACAT-3′],

VGLUT1 (NM_020309.3) [25]

F: 5′-TACACGGCTCCTTTTTCTGG-3′; R: 5′-CTGAGGGGATCAGCAT GTTT-3′], and

β-actin (sequence information unrevealed) [Primer Designed by Bio-Rad (qHsaCED0036269)].

2.5. Statistics

Unpaired two-tailed Student's *t*-tests were used to compare sets of organoid volume and qRT–PCR data. All statistical analyses were carried out using Prism version 7 (Graphpad software Inc., San Diego, CA).

3. Results

3.1. Establishment and growth of human cerebral organoids from ES cells

To analyze the maturation of human cerebral organoids, we established human cerebral organoids from H9 human ES cells as described in Lancaster's protocol [7,8] (Fig. 1, see more details in the methods). This protocol uses only one external growth factor [basic fibroblast growth factor (bFGF)], and the growth of cerebral organoids in this protocol is dependent on the autonomous secretion of growth factors; this protocol is used to mimic the developing embryonic human brain. After establishing the cerebral organoids from human ES cells, we cultured them for up to six months to study the effects of extended culture on these organoids (Fig. 1). ES cells are derived from the inner cell mass in human blastocysts, formed around 5 days after fertilization