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Self-organizing cortex generated from human iPSCs with combination of FGF2 and ambient oxygen

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ABSTRACT

Human brain development has generally been studied through the analysis of postmortem tissues because of limited access to fetal brain tissues. This approach, however, only provides information from the perspective of long-term development. To investigate the pathophysiology of neurodevelopmental disorders, it is necessary to understand the detailed mechanisms of human brain development. Recent advances in pluripotent stem cell (PSC) technologies enable us to establish *in vitro* brain models from human induced PSCs (hiPSCs), which can be used to examine the pathophysiological mechanisms of neurodevelopmental disorders. We previously demonstrated that self-organized cerebral tissues can be generated from human PSCs in a three-dimensional (3D) culture system. Here, we describe the cerebral tissues differentiated from hiPSCs in a further-optimized 3D culture. We found that treatment with FGF2 is helpful to form iPSC aggregates with efficiency. Neuroepithelial structures spontaneously formed with apico-basal polarity in the aggregates expressing forebrain marker FOXG1. The neuroepithelium self-forms a multilayered structure including progenitor zones (ventricular and subventricular zones) and neuronal zone (cortical plate). Furthermore, with the same level of oxygen (O_2) as in ambient air (20% O_2), we found that self-formation of cortical structures lasted for 70 days in culture. Thus, our optimized 3D culture for the generation of cortical structure from hiPSCs is a simple yet effective method.

1. Introduction

Neurodevelopmental disorders, including autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), cause chronic disabilities in the affected individuals. With few effective treatments, individuals with these disorders suffer a great deal, socially and emotionally. To establish new therapies, it is necessary to investigate the pathophysiology of neuro-developmental disorders. Several neurodevelopmental disorders appear to be related to an aberrant cortical structure or dysfunction of neural networks during embryonic brain development. To date, animal models of the brain have provided insight into neural development, but they are limited by their lack of complexity relative to the human brain. Indeed, we still have a very primitive understanding of how human brains develop and function [1].

Recent progress in stem cell biology, combined with basic knowledge of brain development, has led to a three dimensional (3D) culture method that recapitulates brain development *in vitro*. Human pluripotent stem cells (PSCs)-derived 3D structures, referred to as 'brain organoids', have the potential to shed light on key aspects of development. 3D cortical structures have been generated from human iPSCs using several culture protocols [2–4]. However, some difficulties exist among the current protocols, such as the need for special equipment (bioreactor or incubator for high levels of O_2) or tissue manipulation in Matrigel [5,6].

We previously established a 3D culture of human PSC aggregates that recapitulates early development of corticogenesis [or serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq)] [7]. This method has been utilized to generate stratified cortical tissues with apico-basal polarity from hPSCs [8–10]. In this self-organizing culture, PSCs spontaneously form cell aggregates expressing neural progenitor markers, such as NESTIN, PAX6, and SOX2. Following culture under conditions of 40% O_2, PSC-derived neural progenitors make neuroepithelium (NE) in the cell aggregate and then develop cerebral cortex-like tissues

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expressing forebrain markers such as FOXG1, PAX6, and EMX1. The cortical tissues show a laminar structure as seen in the fetal cortex at the second trimester in humans [8,10].

Here, we found that FGF2 is helpful to generate cortical tissues from iPSCs in the SFEBq culture with efficiency. We report that (1) FGF2 is valuable for making iPSC aggregates spontaneously, and (2) FGF2 is useful for differentiation under ambient air conditions (20% O_2) but not 40% O_2. The cortical tissues derived from hiPSCs successfully recapitulated neurodevelopment of cerebral cortex, including the formation of ventricular zone (VZ), cortical plate (CP) and subventricular zone (SVZ). Thus, our culture system supports differentiation of hiPSCs into cortical tissues with reliability and efficiency. These findings will contribute to an understanding of human brain development using hiPSC-derived organoids and may shed light on neurodevelopmental disorders.

2. Materials and methods

2.1. Maintenance of hiPSCs

The 201B7 control hiPSCs were kindly provided by Dr. Yamanaka [11]. hiPSCs were maintained as described previously [12]. Cells were cultured on SNL feeder cells and maintained in hiPSC medium containing DMEM/F12+GlutaMAXI, 20% KnockOut Serum Replacement (KSR), Non-Essential Amino Acid (NEAA) (Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (2-ME) (Sigma), and 5 ng/ml FGF2 (Wako) in a 5% CO_2 incubator. The experimental protocol for dealing with human subjects was approved by the institutional review board at RIKEN.

2.2. Generation of self-organized cortical tissues from hiPSCs

Cortical differentiation from hiPSCs using SFEBq culture was performed as described previously [8] with slight modification. Briefly, the dissociated hiPSCs were quickly re-aggregated with a 96-well V-bottomed culture plate (PrimeSurface MS-9096V; Sumitomo Bakelite) in cortical differentiation medium containing Glasgow's MEM, 20% KSR, NEAA, 0.1 mM 2-ME, 1 mM sodium pyruvate (Thermo Fisher Scientific), and 20 μM Y-27632 (Calbiochem). 3 μM IWR-1-endo (Calbiochem) and 5 μM SB431542 (Tocris) were added to culture from day 0 to day 18. Half of the media was changed every 3–4 days. ROCK inhibitor Y-27632 was removed on day 6 after the SFEBq culture was started (day 0). Recombinant human FGF2 (5 ng/ml, R&D systems) was added on day 3. On culture day 18, floating cell aggregates were transferred to a EZSPHERE dish (IWAKI) in DMEM/F12 with GlutaMAXI, N2 supplement, chemically defined lipid concentrate (Thermo Fisher Scientific) and further cultured in suspension under 5% CO_2/20% O_2 conditions. On day 35, 10% FBS, 5 μg/ml heparin, and 1% Matrigel (growth factor-reduced) (Corning) were added to the medium. On day 56, the tissues were transferred to an oxygen-permeable dish (lummox, SARSTEDT). Cortical tissues were cut in half every 2 weeks from day 35 onward.

2.3. Analysis for the percentage and the diameter of cell aggregates

For Fig. 1B, the number of remaining aggregates were counted every 2 or 3 days during days 18–35. The aggregates with diameters less than 200 μm were excluded. The total number of aggregates at culture day 18 in each experiment was defined as 100%. The diameter of each aggregate was measured at the long axis using ImageJ (NIH). The aggregates were classified into three groups depending on their diameter as follows: large size: >1300 μm, middle size: 700–1300 μm, and small size: < 700 μm.

2.4. Quantitative PCR

Quantitative PCR was performed using the 7500 Fast Real Time PCR System (Applied Biosystems) and data were normalized with the β-actin expression. The primers were as follows: *β-actin*-F: CAATGTGGCCGAGGACTTTG, *β-actin*-R: CATTCTCCTTAGAGAGAAGTGG, *SOX1*-F: GAACGCCTTCATGGTGTG, *SOX1*-R: CTGATCTCCGAGTTGTGCAT, *FOXG1*- F: AGAAGAACGGCAAGTACGAGA, *FOXG1*-R: CGGGTCCAGCATCCAGTAG, *PAX6*-F: TTCACATCTGGCTCCATGTT, *PAX6*-R: GGGTTGCATAGGCAGGTTAT, *EMX1*-F: GTCCGAGCAGAAGAAGAAGG, *EMX1*-R: AGTCATTGGAGGTGACATCG. The values shown on graphs represent the mean ± SD. For quantitative analysis, 8–16 aggregates were examined for each experiment, which was performed in triplicate.

2.5. Immunohistochemistry

Immunohistochemistry was performed as described [13]. Cell aggregates were fixed in 4% PFA/PBS for 45–60 min and cryoprotected in 20% sucrose/PBS overnight at 4 °C. Fixed aggregates were embedded in OCT compound (Sakura FineTek). The tissue cryosections (thickness 10 μm) were transferred to MAS-coated glass slides. Then they were washed in PBS, permeabilized with 0.3% Triton X-100 for 20 min, blocked with 10% normal donkey serum in 0.3% Triton X-100/PBS for 60 min, and incubated with primary antibody at 4 °C overnight. Primary antibodies were diluted with 0.05% Tween20/PBS at the following dilutions: FOXG1 (1:1,000, custom, [14]), NESTIN (1:200, PRB-570C, Covance), COUP-TF1 (1:1000, PP-H8132-00, Perseus Proteomics), SP8 (1:500, sc-104661, Santa Cruz), TUJ1 (1:500, MMS-435P, Covance), SOX2 (1:250, sc-17320, Santa Cruz), PAX6 (1:250, PRB-278P, Covance), TBR1 (1:1000, ab31940, abcam), CTIP2 (1:5000, ab18465; abcam), TBR2 (1:500, AB9618, Chemicon), SATB2 (1:75, ab51502, abcam), PKCζ (1:100, sc-216-G, abcam). Fluorescence-tagged secondary antibodies were as follows: Alexa Fluor 488- (1:500), Cy3- (1:400) and Cy5- (1:400), conjugated IgG (Jackson ImmunoResearch). DAPI was used for counterstaining the nuclei (Molecular Probes). All immunohistochemical images were acquired using confocal microscope (Carl Zeiss, LSM710).

2.6. Statistical analysis

All data are shown as mean ± SD. Statistical analyses were performed with PRISM software (GraphPad, version 6). Statistical significance was tested by unpaired Mann-Whitney test (nonparametric) for two-group comparisons or Kruskal-Wallis with Dunn's test for multi-group comparisons.

3. Results

3.1. FGF2 for efficient self-organization of hiPSC-derived cortical tissues

It was previously demonstrated that human embryonic stem (ES) cells (hESCs) can be induced to differentiate into cortical tissues when cultured as floating aggregates (typically 9000 cells per aggregate) in SFEBq culture [8,10]. However, mESC-derived cortical tissues are generated from a small number of cells (typically 3000 cells per aggregate) in SFEBq [14]. To compare characteristic features of hiPSC-derived cortical tissues that originated from different-sized cell groups, we cultured hiPSCs at a density of 12,000 or 5000 cells per well in SFEBq culture. We found that shapes of the cell aggregates were morphologically different, depending on the cell number. On day 19, the whole size of each aggregate induced from 5000 cells/well was smaller than the size of

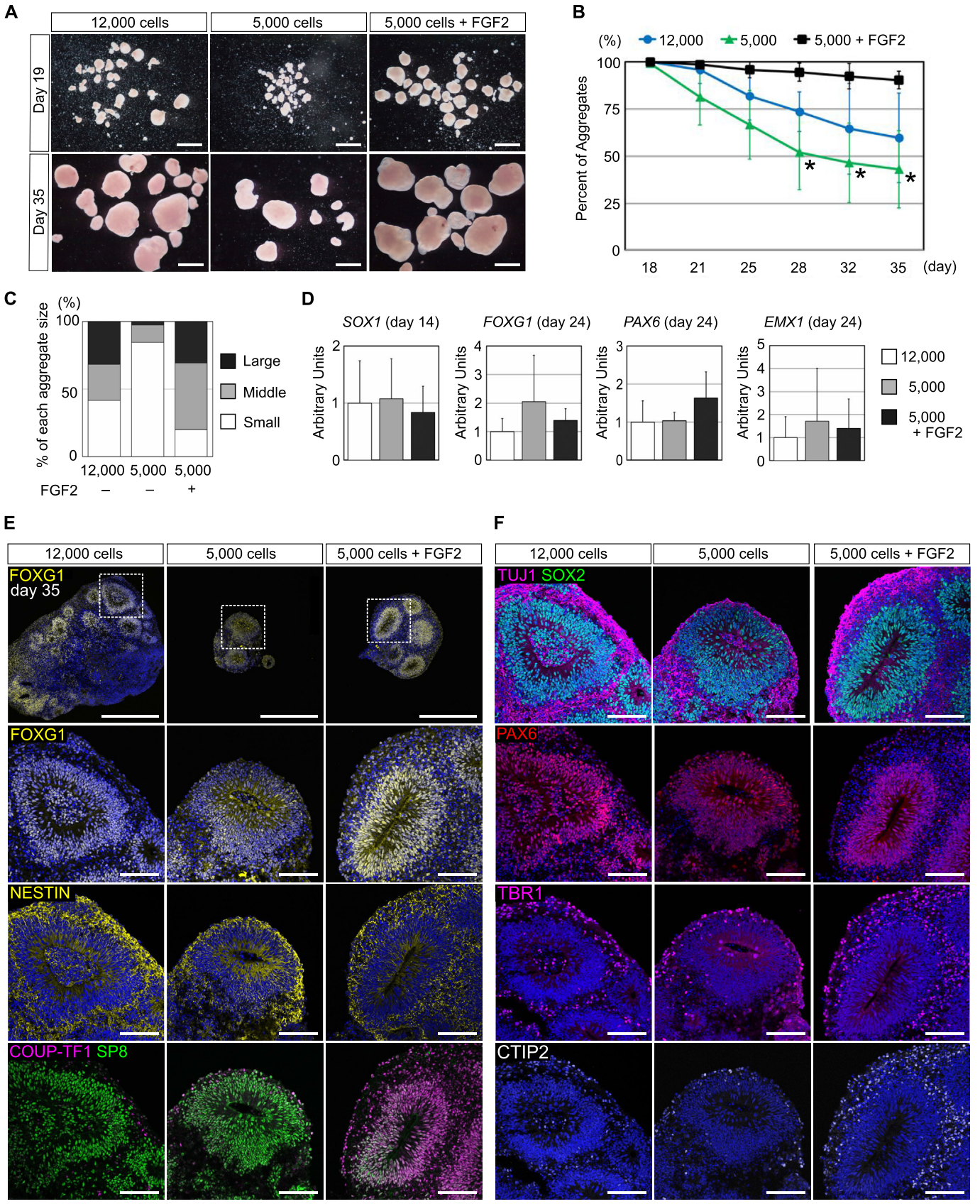

Fig. 1. Comparison of iPSC-derived cortical tissues. (A) Appearance of cell aggregates in bright field at day 19 and 35. (B) Percent of aggregates that could be observed from day 18–35. The total number of aggregates at culture day 18 in each experiment was defined as 100%. Asterisks represent significant differences in each value between the FGF2-treated and -untreated aggregates. $^{*}P < 0.05$. (C) Ratio of the diameter of cell aggregates. The sizes mean: Large: > 1300 μm, Middle: 700–1300 μm, Small: < 700 μm. Aggregates from three

each aggregate induced from 12,000 cells/well (Fig. 1A). The aggregates generated from 5000 cells/well were fragile and gradually broke up during culture, decreasing at a much faster rate than the cell aggregates generated from 12,000 cells/well (Fig. 1B). To enable the formation and growth of cell aggregates, we applied FGF2, which promotes neurogenesis in culture [9,15], in the 5000 cells/well SFEBq culture. As expected, addition of FGF2 (5 ng/ml) on culture day 3 led to the formation of cell aggregates and support their growth (Fig. 1A and B) (condition 1 in Supplementary Fig. 1A). Addition of FGF2 (5 ng/ml) on day 6 failed to support the growth (Supplementary Figs. 1A–C). Treatment with FGF2 (5 ng/ml) during days 3–18 and 20 ng/ml on day 3 induced the formation of cell aggregates (Supplementary Figs. 1A–C), but not much *FOXG1* expression was observed on day 24 (Supplementary Fig. 1D). Since FOXG1 expressing neuroepithelial structures appeared in the cell aggregate treated with FGF2 (Supplementary Fig. 1E), we assume that application of FGF2 (5 ng/ml) on day 3 had a strong effect on the formation of neuroepithelial structures originating from the small number of hiPSCs (5000 cells/well) in SFEBq culture. After treatment with FGF2, cell aggregates grew to a larger size. The appearance of small-sized aggregates (diameter shorter than 700 μm) decreased and middle-sized aggregates (700–1300 μm) increased on day 35 (Fig. 1C). These results suggest that FGF2 could promote the formation and development of cell aggregates.

Next, we examined the expression levels of the early neural progenitor marker (*SOX1*) and the telencephalon-specific markers (*FOXG1*, *PAX6*, and *EMX1*) in qPCR analysis. It is known that FGF2 influences cell proliferation and neural differentiation in brain development [16], however, we did not observe significant differences in the expression of these markers when the FGF2 treatment started on day 3 (Fig. 1D). On day 35, immunohistochemical analysis showed that FOXG1 was expressed in the NESTIN$^+$ neuroepithelial structure generated from FGF2-treated 5000 cells as seen in FGF2-non-treated 12,000 cells (Fig. 1E). Interestingly, even transient FGF2 treatment (day 3 onwards) was sufficient to substantially induce COUP-TF1 expression, which is expressed in the caudal region of cerebral cortex. In contrast, the expression of SP8, a marker for rostral cortex, decreased when FGF2 added on day 3 (Fig. 1E). FGF2 has a weak caudalizing activity [13]. These results suggest that transient treatment with FGF2 caused moderate caudalizing of cell identities in hiPSC-derived cortical tissues, consistent with a previous study [13]. We examined expression patterns of specific markers for cerebral cortex using immunohistochemical analysis. FGF2 treatment was helpful to form a multilayered structure in hiPSC-derived cortical tissues. SOX2$^+$ and PAX6$^+$ progenitor cells were observed inside the cell aggregates, as seen in the VZ of cortex. TUJ1$^+$ neurons were observed at the outside (Fig. 1F). TBR1 and CTIP2, markers for neurons in the cortical plate, were expressed in the TUJ1$^+$ layer, suggesting that these cells differentiated into cortical neurons in a deep layer. These results suggest that FGF2 is valuable for efficiently generating cortical tissues in SFEBq culture.

3.2. Effects of FGF2 on generation of hiPSC-derived cortical tissues cultured under ambient air conditions

We found that generation of cortical tissues does not require a large number of hiPSCs in the presence FGF2 (day 3 onwards) in SFEBq culture. FGF2 is effective for recapitulation of cortical structure in cell aggregates generated from a small number of cells (5000 cells/well). In addition, we observed that the middle-sized aggregates (700–1300 μm in diameter) increased in culture when the FGF2 treatment started on day 3 (Fig. 1C). In SFEBq culture described previously, cell aggregates were incubated under hyperoxic (40% O_2) conditions from day 18 to enable the growth and long-term survival of hPSC-derived cortical tissues [8]. Next, we examined the effects of combining FGF2 and ambient air conditions (20% O_2) for generation of cortical structure.

We cultured hiPSC aggregates under 20% O_2 or 40% O_2 from day 18 after the addition of FGF2 (day 3 onwards). We did not observe clear changes under a dissecting microscope for cell aggregates cultured in a 20% O_2 incubator (Supplementary Fig. 2A). Most of the cell aggregate was maintained in a healthy manner under 20% O_2, as seen under hyperoxic conditions (Supplementary Fig. 2B). In qPCR analysis, we did not observe significant differences in expression of *FOXG1, PAX6* or *EMX1* on day 24 (Supplementary Fig. 2C). On day 35, immunohistochemical analysis showed that the NE structures of hiPSC aggregates cultured in 20% O_2 were similar to those seen in the 40% O_2 condition (Fig. 2A compare to Fig. 1E and F). As expected, the appearance of middle-sized tissue (700–1300 μm in diameter) increased when hiPSC aggregates were cultured in 20% O_2 (Fig. 2B). The middle-sized cortical tissues showed layered structures with apico-basal polarity, that is, PAX6$^+$ and SOX2$^+$ cells located near the PKCζ$^+$ apical side (ventricular zone; VZ) and TUJ1-, TBR1-, CTIP2-positive cells located at the basal side at outside (cortical plate; CP) in the aggregate (Fig. 2C). We did not observe any significant differences in thickness of VZ and CP between the 20% O_2 condition and the 40% O_2 condition (Fig. 2D). These results suggest that the addition of FGF2 supports the maintenance of hiPSC-derived cortical tissues under ambient air conditions (20% O_2). The hyperoxic (40% O_2) condition is not required for cortical generation in the presence of FGF2.

3.3. Long-term culture of hiPSC-derived cortical tissues

Finally, we examined formation of cortical structure after long-term culture in the combined FGF2 and 20% O_2 condition. In the presence of FGF2, hiPSC aggregates grew to a large size, around 1 mm in diameter on day 35, in the 20% O_2 condition. About 40–50% of hiPSC aggregates formed a cortex-like structure by day 56 (data not shown). The formation of VZ (PAX6$^+$, SOX2$^+$) and CP (TUJ1$^+$, TBR1$^+$, CTIP2$^+$) were clearly observed in hiPSC-cortical tissue (Fig. 3A). The thickness of CP was significantly increased on day 56 compared to day 35, whereas the VZ was not altered (Fig. 3B).

On day 70, hiPSC aggregates with cortex-like structures decreased in culture, to about 20–30% of the total aggregates (data not shown). Immunohistochemical analysis showed that TBR2$^+$ cells were observed in the layer between VZ (SOX2$^+$/PAX6$^+$) and CP (TBR1$^+$/CTIP2$^+$), indicating intermediate progenitor cells appeared in the SVZ (Fig. 3C). SATB2, a marker for cortical neurons in the upper layer, was expressed in CP on day 70 (Fig. 3C). These observations suggest that the combination of FGF2 and 20% O_2 in SFEBq culture is effective for the generation of hiPSC-derived cortical tissues.

4. Discussion

In the present study, we demonstrated that FGF2 is valuable for the generation of cortical tissue from hiPSCs in SFEBq culture. Of note, even transient FGF2 treatment (day 3 onwards) was sufficient to substantially induce the formation of FOXG1$^+$/NESTIN$^+$ NE

distinct experiments were measured. (D) Expression of mRNA relative to the expression in 12,000 cells/well (n = 6). (E, F) Expression pattern of dorsal telencephalon markers. Dotted squares indicate magnified fields in E and F. Scale bars, 1 mm (A), 500 μm (top panels in E), and 100 μm (panels except top in E, F). See also Fig. S1.

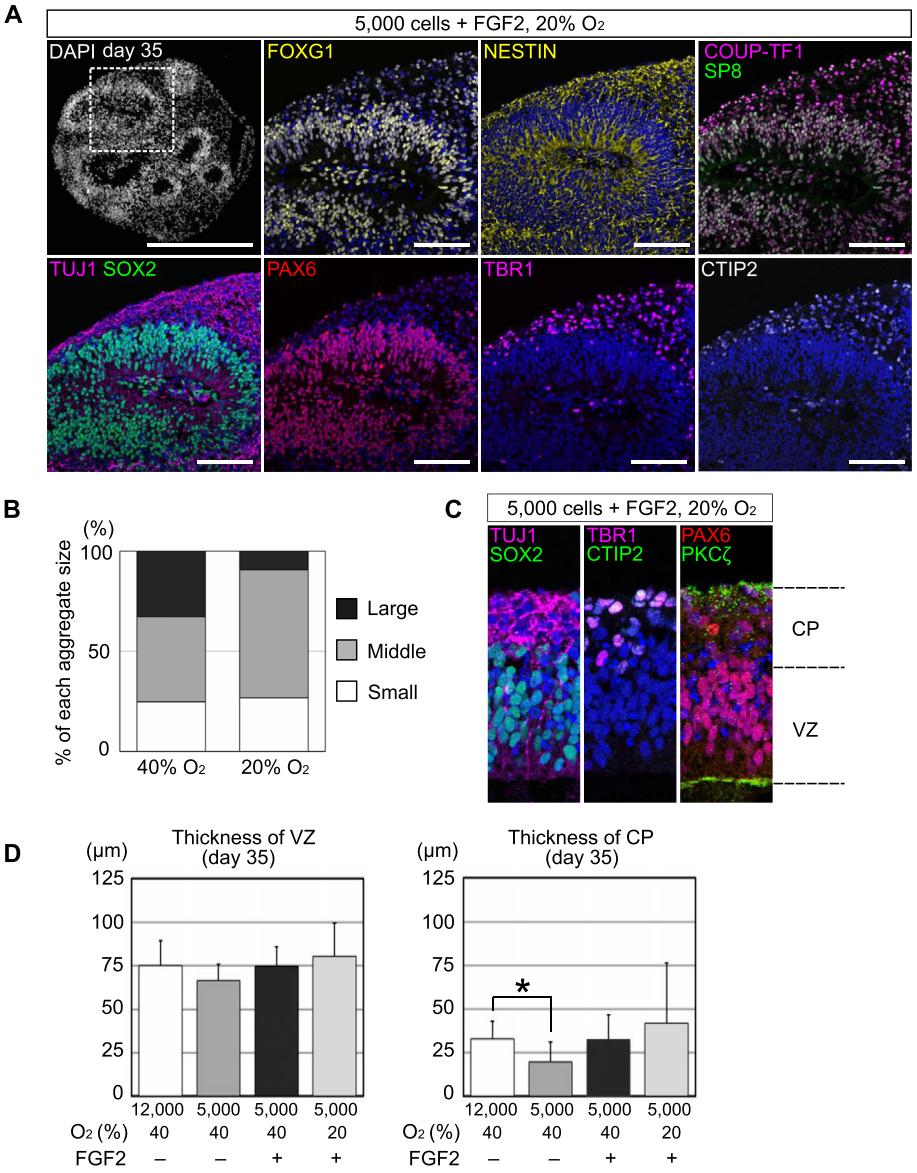

Fig. 2. Differentiation of iPSCs into cortical tissues with FGF2 in 20% O_2. (A) Expression pattern of cortical neural progenitor markers. Insets in the DAPI-stained panel indicate magnified image. Scale bars, 500 μm (panel stained with DAPI) and 100 μm (other panels in A). (B) Ratio of the diameter of cell aggregates. The sizes mean: Large: > 1300 μm, Middle: 700–1300 μm, Small: < 700 μm. Aggregates from three distinct experiments were measured. (C) The layered structures observed in the aggregate on day 35. (D) Thickness of VZ- or CP in cortical layered structures on day 35. $^*P < 0.05$. See also Fig. S2.

structure. FGF2 enabled hiPSC aggregates to grow according to early development under ambient air conditions (20% O_2) but not hyperoxic conditions (40% O_2). Therefore no special equipment such as a multi-gas incubator is required for generation of cortical structure from hiPSCs. These findings will facilitate research related to human brain development using hiPSCs.

In SFEBq culture, providing oxygen is necessary for generation of cortical tissues from hPSCs [8,10]. However, it has been reported that embryonic stem and progenitor cells are exposed to hypoxic niches and low O_2 regulates their differentiation [17,18]. To investigate human brain development, it is best to differentiate hPSCs according to the development as seen *in vivo*. In this view, it is reasonable that hPSCs are cultured under hypoxic conditions (e.g. 2–9% O2, refer to [17]). In the present culture system, the concentration of O_2 (20%) is still hyperoxic compared to embryonic life. We hope to establish conditions closer to those seen in development, although it may require difficult techniques.

It is well known that FGF regulates cell proliferation and cell differentiation during development [16]. In addition, we previously reported FGF2 has a weak caudalizing activity in SFEBq culture [13]. Although we have demonstrated the efficacy of transient FGF2 treatment in SFEBq culture up to day 70, it could not be used to generate mature cortical tissues. Since FGF signaling regulates various developmental steps in the brain, it will be necessary to examine in detail the mechanisms of the supportive effect for the hiPSC-derived cortical formation in the present culture system.

Disturbance of neurodevelopment is thought to lead to some neurodevelopmental disorders [1]. Our method may be used to recapitulate early development of the cerebral cortex, thus allowing researchers to uncover the pathophysiology and mechanisms underlying neurodevelopmental disorders.

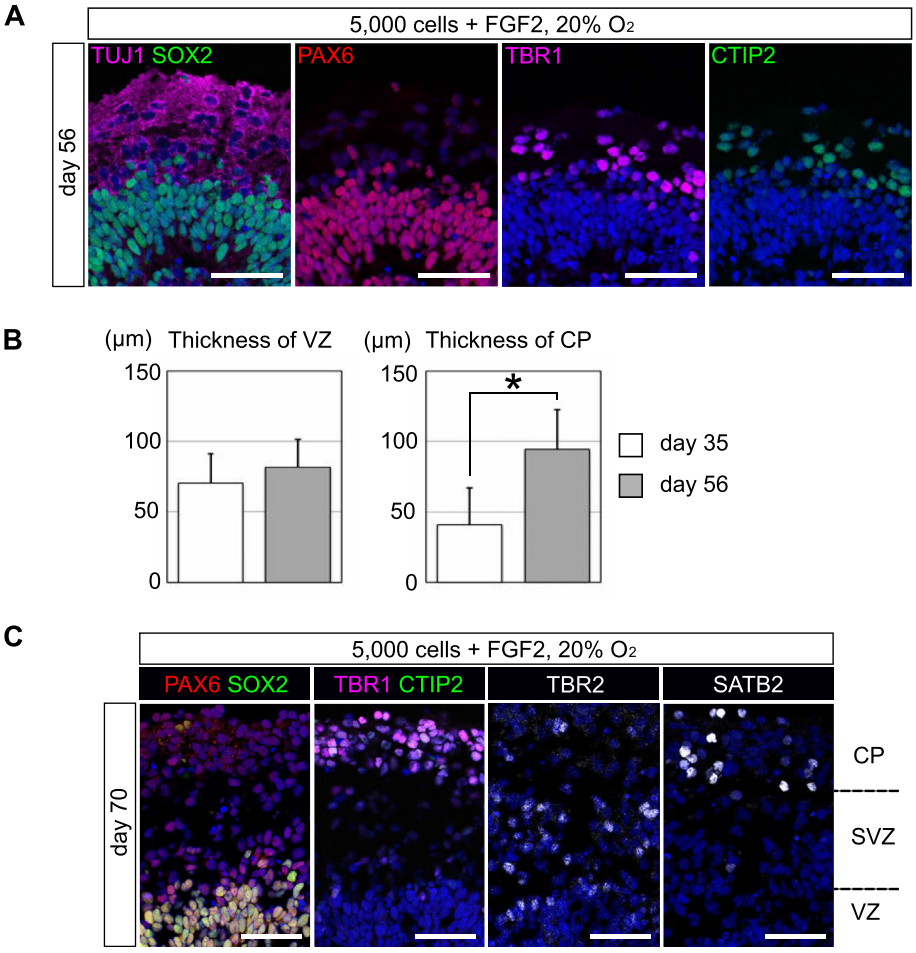

Fig. 3. Layered structures of 56 or 70 days in culture. (A) Expression pattern of marker proteins of VZ and CP. (B) Thickness of VZ or CP on day 35 and 56. (C) Development of NE structures observed on day 70. Scale bars, 50 μm (A, C). $^{*}P < 0.05$.

Author contributions

K.M. conceived and supervised this project. N.E. performed the experiments. K.M. and N.E. performed data analysis. K.M. and N.E. wrote the manuscript. I.S. provided critical reading and scientific discussion. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.03.049.

Transparency document

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