Develop a 3D neurological disease model of human cortical glutamatergic neurons using micropillar-based scaffolds

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Abstract

Establishing an effective three-dimensional (3D) \textit{in vitro} culture system to better model human neurological diseases is desirable, since the human brain is a 3D structure. Here, we demonstrated the development of a polydimethylsiloxane (PDMS) pillar-based 3D scaffold that mimicked the 3D microenvironment of the brain. We utilized this scaffold for the growth of human cortical glutamatergic neurons that were differentiated from human pluripotent stem cells. In comparison with the 2D culture, we demonstrated that the developed 3D culture promoted the maturation of human cortical glutamatergic neurons by showing significantly more MAP2 and less Ki67 expression. Based on this 3D culture system, we further developed an \textit{in vitro} disease-like model of traumatic brain injury (TBI), which showed a robust increase of glutamate-release from the neurons, in response to mechanical impacts, recapitulating the critical pathology of TBI. The increased glutamate-release from our 3D culture model was attenuated by the treatment of neural protective drugs, memantine or nimodipine. The established 3D \textit{in vitro} human neural culture system and TBI-like model may be used to facilitate mechanistic studies and drug screening for neurotrauma or other neurological diseases.
1. Introduction

The human cerebral cortex is the most complicated organ that plays essential roles in learning and memory, emotion, cognitive and motor function\textsuperscript{11,12}. Damage or degeneration of the human cerebral cortex causes severe diseases, for example, traumatic brain injury (TBI)-related cognitive defects. However, the human brain disease model for studying the pathogenesis and treatment is absent. The recent breakthroughs in the study of human pluripotent stem cells (hESC, human embryonic stem cell; iPSC, induced pluripotent stem cells)\textsuperscript{13–15} have provided powerful tools to generate all types of human cells\textsuperscript{9,16}, including human cortical neurons of the cerebral cortex\textsuperscript{17–19}. However, the classic generation and maturation of hPSC-derived cortical neurons on 2D planar coverslip do not accurately mimic the human neural micro-environment. As a result, some essential characteristics of human cortical neurons, such as TBI. Therefore, recreating in vivo physiological characteristics of hPSC-derived cortical neurons is imperative.

The 3D structure provides a better cell growth micro-environment than the 2D planar coverslip. The mRNA levels of functional genes in neuronal adhesion, regenerative growth, and synaptogenesis were much higher in the 3D structure, in comparison with the 2D culture\textsuperscript{20}, suggesting that the scaffold's architecture may improve the cell binding and neuronal outgrowth. Indeed, the capacity of cell adhesion and the formation of a functional structure are enhanced in the 3D culture\textsuperscript{16}. However, there remain unsolved concerns regarding the 3D culture: (1) culturing human-specific neuronal lineage, for example, human cortical glutamatergic neurons\textsuperscript{15,17}, is lacking; (2) 3D cultures for modeling human neurological diseases like TBI have not been well established\textsuperscript{17–19}; (3) drug studies for neurological diseases by using 3D culture have not yet been reported\textsuperscript{15,19}.

Herein, we designed the hPSC-derived human cortical neurons with the brain-like function on a polydimethylsiloxane (PDMS) micropillar surface. Adapted with the structure of these 3D micropillars, the differentiation of human cortical neurons was promoted, and the proliferation of neural progenitors was inhibited, which subsequently resulted in the maturation of a glutamatergic neuron-rich culture system. Furthermore, the mimicking of brain injury and drug tests were attempted on the 3D culture system.

2. Materials and methods

2.1. Construction of PDMS pillars

The scaffold was produced by a one-step casting replication process as previously reported\textsuperscript{15}. The area of the pattern region was 1 cm $\times$ 1 cm. The mold was salinized overnight with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane. The 10:1 mixture of the PDMS base and the curing agent was spread over the salinized silicon mold, and cured at 100 °C for 5 min. After curing, the PDMS layer was broken off from the mold in an isopropanol medium. Then, the PDMS layer was stored in 75% ethanol. After a 24–48 h soaking, the PDMS scaffolds were moved to a cell-culture hood and washed with phosphate-buffered saline for 3 times. Before the cells were seeded into the 3D PDMS scaffold, the device was coated with Matrigel (Discovery Labware, Inc., Bedford, USA) for overnight.

2.2. hPSCs maintenance and passage

Human ESCs (H9, passages 60–80, WiCell Agreement No. 16-W0067) and human iPSCs (IMR90-4, passage 40–50, WiCell Agreement No. 17-W0063) were maintained in a feeder-free environment on vitronectin (Life Technologies, Maryland, USA)-coated plates\textsuperscript{21,22}. A standard protocol using Essential 8 basal medium (Life Technologies, Carlsbad, CA, USA) was previously reported on http://www.wicell.org. hPSC colonies were passaged by using EDTA (Lonzia Inc., MA, USA) when they became 70% confluent.

2.3. Differentiation of cortical glutamatergic neurons from hPSCs

To start the differentiation, hPSCs colonies were treated with 1 U/mL dispase (Life Technologies, MA, USA) at 37 °C for 1–2 min as previously reported\textsuperscript{15}. After detachment, the cells were suspended in neural induction medium (NIM). The colonies were aggregated to shape embryonic bodies (EBs) from day 0 to day 7. At day 7, the EBs were attached to 6-well plates for neural rosette formation. The rosette structure was observed at day 10. At day 16, the rosettes were detached by a 1000 µL pipette and resuspended in NIM containing B27 (Life Technologies, New York, USA), which may reduce the damage caused by the mechanical pipette. At day 17, the cells formed neural spheres (NSs). At day 24, NSs were dissociated with TrypLE (Life Technologies, CA, USA) and seeded onto a coverslip pre-coated with Matrigel at a density of 40,000 cells per coverslip in 12-well plates. After 2 h, 1.5 mL pre-warmed NIM containing B27 was added into each well. Moreover, NSs were also dissociated into 3D scaffolds pre-coated with Matrigel at a density of 35,000 cells/cm² in 6-well plates. After 2 h, 1.5 mL pre-warmed NIM containing B27 was added into each well. The medium was refreshed every 7 days.

2.4. Quantitative real-time polymerase chain reaction (qPCR)

Cells were collected at day 28 and dissociated with Trizol reagent. The RNA was extracted from neurons and diluted to 1 µg with DEPC-treated water. SuperScript III First-Strand system (Life Technologies, CA, USA) was used to synthesize the cDNA. qPCR analysis was performed in the 20 µL reaction system, which consisted of 4 µL cDNA, 2 µL random primers, 2 µL dH$_2$O and 10 µL 2X SYBR Green RCR Master Mix (Roche Molecular
2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C. Then the cells were washed with phosphate-buffered saline (PBS) for 5 min. After being washed with PBS for 3 times, the cells were processed with 0.2% Triton X-100 for 10 min and then 10% donkey serum for 1 h at room temperature. The cells were incubated with primary antibodies, which were in 0.1% Triton X-100 and 5% donkey serum at 4°C for overnight. On the next day, the cells were washed with PBS for 10 min. After being washed with PBS 3 times, secondary antibodies were prepared in 5% donkey serum. Moreover, the samples were incubated for 30 min at room temperature, avoiding light during the whole process. The nuclei were counterstained with Hoechst 33258 (Molecular Probes, Inc, Oregon, USA). Then, the cells were washed with PBS for 10 min. After being washed with PBS 3 times, secondary antibodies were prepared in 5% donkey serum. Moreover, the samples were incubated for 30 min at room temperature, avoiding light during the whole process. The nuclei were counterstained with Hoechst 33258 (Molecular Probes, Inc, Oregon, USA). Then, the cells were washed with PBS for 3 times 10 min, and mounted for fluorescent imaging. Cell death was detected using TUNEL assay (Vazyme biotech co., Ltd., Nanjing, China), following the manufacturer's protocol. Antibodies are listed in Supporting Information Table S2.

2.6. Glutamate concentration measurement

Approximately 50 μL of collected D45 neuron medium was added to the 100 μL reagent. After incubation at 37°C for 30 min, the sample was added to 96-well plates (50 μL/well). The glutamate concentration was detected by using a microplate reader (Molecular Devices Spectra Max M2, Molecular Devices, CA, USA). Reagents were obtained from Sigma Technology. The stock solution of nimodipine and memantine was 10 mmol/L in DMSO. These two compound solutions were stored in –80°C, and the working concentration of nimodipine and memantine was 100 mmol/L. Nimodipine was added 3 h ahead of injury, and memantine was added 10 h ahead of injury.

2.7. Establishment of brain injury-like model

A custom-made weight-drop setup (a 7.5 g weight and a 15 mL tube for the height) was used for inducing injury. Nimodipine was added 3 h, and memantine was added 10 h before the blunt hit. Solution samples were collected (150 μL) before and after injury for analysis.

2.8. Statistical analysis

To quantify the results, a fluorescence microscope (Nikon Eclipse 80i, New York, USA) was used to capture the images. At least 12 random fields were chosen, and more than 5000 cells of each cell line were quantified, and at least 3 independent differentiation assays were performed. The images were constructed using Adobe Illustrator CC. Values were obtained using paired t-test with Image-J software (National Institutes of Health, MA, USA). The data are presented as SEM, and P-value < 0.05 was considered significant.

3. Results

3.1. 3D PDMS pillars promoted the differentiation of hPSC-derived neurons

An array of PDMS pillars were designed to perform the neural growth and differentiation. Human embryonic stem cell (hESC)-derived neural progenitors (H9) at differentiated day 24 (D24) were dissociated by TrypLE, and seeded on Matrigel-coated 3D PDMS with these soft pillars (Fig. 1A–B). For comparison, the cells were cultured on 2D coverslip and 2D (planar) PDMS surface as “2D PDMS group”. At day 28 (D28), the expressions of REST, the marker of neural stem cells, and Ki67, the marker of dividing cells, were decreased in the 3D PDMS group. Moreover, the expressions of DCX, the marker of newly born neurons, and TUJ1, the marker of neurons, were increased in the 3D PDMS group (Fig. 2A–C). qPCR analysis of cortical neurons (TBR1 and CTIP2) and mature neurons (NEUN and MAP2) indicated that 3D PDMS pillars promoted the differentiation and maturation of cortical neurons (Fig. 2D). To exclude the possible discrepancy of the dorsal forebrain identity on these two surfaces, the cells were labeled with PAX6, the marker of cortical progenitor cell, and FOXG1, the marker of forebrain progenitor cell (Fig. 2E). Similar percentages of PAX6 and FOXG1 were obtained, which indicated that the 3D PDMS surface did not change the cell fate of dorsal forebrain. These results suggested that the 3D PDMS structure inhibited the cell proliferation and promoted...
the neural differentiation that derived from hPSCs. One additional cell line, induced pluripotent stem cell line IMR 90-4, was used to test the effect of the 3D culture environment on neural proliferation and maturation (Fig. 2F). Similar results were observed between H9 hESC and IMR90-4 hiPSC-derived neurons on the 3D culture. At day 35 (D35), the expression of KI67 further decreased, and the expression of MAP2, the marker of mature neurons, increased in the 3D PDMS group (Fig. 2G).

### 3.2. The density of PDMS pillars affects the neural maturation

To further determine whether the density of PDMS pillars affected human neural differentiation and maturation, we plated the human cortical progenitors on dense and sparse PDMS surfaces, respectively (Fig. 3A–B). In the dense region, the distance between pillars is from 3.18 to 6.55 μm. Whereas in the loose region, the distance between pillars is from 8.14 to 13.7 μm (Fig. 3A–B). At the
day 35 (D35), the proportion of dividing cells, differentiated neurons and mature neurons were quantified. The percentage of KI67 and TUJ1 positive cells among total cells were approximately 25% and 40%, respectively (Fig. 3C). Interestingly, more MAP2 positive cells were observed on the 3D PDMS surface with dense pillars (Fig. 3C), suggesting that the possible reason is that denser pillars may provide more binding sites for neurons, which in turn creates a more stable microenvironment for neurites growth to be more mature. Altogether, the 3D PDMS structure promoted neuronal differentiation of neural progenitor cells and the maturation of neurons. Furthermore, the higher density of PDMS pillars enhanced the maturation of hPSC-derived neurons.

3.3. 3D PDMS pillars promoted the maturation of hPSC-derived cortical cells

The generation of cortical neurons is crucial during the development of the human brain. Therefore, we next determined the generation of cortical neurons in the 3D PDMS group. At day 42 (D42), the expression of TBR1 (the T-box homeobox protein, localized in SP and CP, and later in layer V) and the superficial-layer cortical marker CTIP2 (the transcription factor related to specifying subcortical projection neurons) were significantly increased in the 3D PDMS group (Fig. 4A). Notably, SATB2, the marker of cortical neurons in upper layers, was remarkably expressed in the 3D PDMS group, while it was barely found in the 2D group at day 42 (D42) (Fig. 4A). The results indicated that the 3D PDMS pillars promoted the generation of cortical neurons, compared to the 2D planar culture (Fig. 4B).

3.4. 3D PDMS pillars promoted the maturation of human neurons

Since the generation of more mature neurons favored the production of a more complex neuronal network, the maturation-related marker of neurons in the 3D PDMS group was tested. At day 42 (D42), the primary branch was measured. The result showed that the primary branch was significantly increased in the 3D PDMS group (Fig. 4C). At day 63 (D63), the density of SYNAPTOPHYSIN+ puncta that localized along the neurites was higher in the 3D PDMS group (Fig. 4D). Significantly, an average of 8 SYNAPTOPHYSIN+ puncta per 100 μm neurites in the 3D PDMS group was observed, compared to the 4.5 SYNAPTOPHYSIN+ puncta per 100 μm neurites in the 2D group (Fig. 4D–E), which
suggested that the 3D PDMS pillars promoted the maturation of the cortical neuronal network. The confocal image of D63 neurons indicated that a 3D neuronal structure had been formed, instead of the single layer of cortical neurons (Fig. 4F).

3.5. Traumatic brain injury-like model and drug testing

Finally, to find out whether the human neuron 3D culture could model human brain disease in vitro, we designed a brain-injury cell disease model to test the application of the human cortical neurons in the 3D PDMS culture. To model the brain injury, we used a blunt object for inducing the cell injury (Fig. 5A). To test the reliability of the dropping model, we compared the expression of Caspase3 and TUNEL before and after the dropping. The result showed that the dropping increased the expression of Caspase3 and TUNEL, which suggested that the disease modeling is successful (Fig. 5B). Glutamine levels were increased dramatically 8 min after the dropping (Fig. 5C), which was consistent with the in vivo study23, suggesting that the in vitro culture modeled the glutamine release after the injury.

Nimodipine is a calcium channel blocker used to protect the neuronal cells in clinical conditions24. We pretreated the neurons with 100 nmol/L nimodipine 3 h before stimulation, and the glutamine level was significantly reduced after 8 min post-dropping, compared to the untreated group (Fig. 5C), suggesting that the nimodipine may maintain the glutamine levels to protect neurons during injury. The other US Food and Drug Administration (FDA)-approved medicine, memantine, that is an antagonist of the excitatory amino acid receptor25, was tested on the 3D hPSC-derived neurons. Similarly, the glutamine levels were reduced significantly during cell injury when pretreated with 100 nmol/L memantine (Fig. 5D). Taken together, our results suggested that the 3D PDMS culture system provided a dynamic human neuron model which may be useful for the drug study of brain-injury-related diseases like TBI or craniofacial injury.
4. Discussion

In this study, we built the PDMS scaffold to model the neural 3D culture micro-environment. Our results suggested that the 3D PDMS scaffold promoted the neural differentiation and maturation of hPSCs. Furthermore, the generation of cortical neurons was also enhanced in the 3D PDMS group. Chitin-alginate 3D microfibrous scaffolds18 and Donut-like 3D material15 were reported as efficient materials to promote the differentiation and maturation of rodent neuronal cells. Similarly, our study showed that the 3D PDMS pillars accelerated the maturation of hPSC-derived cortical neurons. Further, we used our 3D culture system to test the physiological response of human cortical neurons, and found it to be close to that in in vivo brain injury. Therefore, our system provided a dynamic 3D model to study the human cortical neurons.

Compared to traditional 3D cultures, PDMS is easy to make and get autoclaved in the lab, and is biocompatible. PDMS has low attachment for the cell growth, and it has also been confirmed that PDMS has no negative effect on the neuronal differentiation. In this study, we promoted the efficiency of neuronal differentiation and maturation by using 3D PDMS scaffolds. Generation of human cortical neurons usually took a long time in vitro12,13. For example, SATB2 is the marker for the upper layer of cortical neurons, and is expressed relatively late. In the traditional 2D planar culture, SATB2 was not detected until at least ten weeks (70 days) of differentiation13. In contrast, we found that the SATB2 was largely expressed in our 3D culture system as early as day 42 of differentiation, suggesting that our current culture system enhanced the generation of human cortical neurons in vitro.

Our 3D human cell culture model is vital for studying many human CNS diseases, which is inaccessible in 2D cultures26. To improve the use of 3D scaffolds for drug screening and drug delivery purposes, it is required to alter the chemical and biophysical properties of the materials to better suit the needs of the cultured cells and native tissues27. Taking traumatic brain injury (TBI) as an instance, we showed that human cortical neurons responded to physiologically relevant impact forces. The 3D cell model exhibited increased injury-induced glutamine release that was consistent with in vivo studies15. Furthermore, with the treatment of two brain protective medicines, the glutamine-release was significantly reduced after the TBI-like simulation. Therefore, our 3D culture system provided a dynamic model for traumatic brain injury in vitro.

5. Conclusions

Taken together, all of the results showed that our 3D PDMS pillar-based cell model enhanced the generation of human cortical glutamatergic neurons. Furthermore, the feasibility of a 3D PDMS culture system was successfully tested by the brain protective medicines, nimodipine and memantine. After an 8 min period of brain injury-like stimulation, the glutamine-release was significantly reduced with the treatment of these two brain protective medicines. We provided a dynamic model for traumatic brain injury in vitro.
injury treatment testing in vitro, which might be applied to neurological studies and drug screening.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2019.03.004.

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