Progress in human pluripotent stem cell-based modeling systems for neurological diseases

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ABSTRACT
Human pluripotent stem cell (hPSC)-based modeling offers the potential for studying human diseases using human systems. An increasing number of studies in numerous fields demonstrate that hPSC-based disease systems capture disease specific pathophysiology occurring in vivo. A widespread deployment of hPSC systems is foreseeable. Even the field of psychiatric disorders (for example, schizophrenia and autism), which lags behind due to complex underlying causes, such as the inaccessibility of brain cells for assessments and the absence of reliable models, has been embracing the hPSC-based disease system. However, despite hPSCs holding great potential, it is imperative to validate how faithful hPSC-based neural developmental modeling is in recapitulating the developmental process in vivo. Our recent study demonstrated that the hPSC-based system mimicked the process of neural development and the system reserved neural stem cell (NSC) niches similar to those residing in the ventricular region of the cortex. In this article, we will first comment on an array of factors that affect hPSC-based neural differentiation and summarize the intricate regulatory signaling pathways that regionalize neuronal cell types. Finally, we review successful studies in brain-related diseases using hPSC-based modeling with 3-D systems.

KEYWORDS
3-D culturing; cell fate decision; hPSC-based disease modeling; human pluripotent stem cell; neural developmental modeling

Before the discoveries of human pluripotent stem cells (hPSCs), scientists primarily used animal modeling systems to study human diseases. However, it became apparent that in most cases animal models cannot faithfully recapitulate human diseases. Some systematic reviews in animal modeling pointed out the poor quality of animal research and the difficulty in extrapolating from animal data to humans. With the advent of hPSC technologies, the scientific community sees modeling human diseases in a different light. Researchers have been harnessing and adopting the power and properties of hPSCs to investigate human disorders using human systems. The potential of PSCs as disease modeling systems has been widely received in many fields judging by the exponential increase in publications. For example, in cancer, cardiovascular, Diabetes Mellitus, eyes, the prostate and bladder, blood, and brain-related diseases. We are stem cell biologists with pragmatic views and we believe in the value hPSC-based systems can bring, not only to disease research fields but also to developmental biology.

In this article, we comment on our observations while establishing hPSC-based neural developmental modeling systems. There are numerous factors that affect the neural differentiation of hPSCs. They include coating materials, sources of materials, ways of handling, the density and form (single-cell or clump) of plating out, methods of preparing cells (enzymatic, EDTA, or mechanical), the timing and protocols of differentiation induction, and culturing systems (e.g. 2-D, 3-D, or EBs). Although a myriad of factors determining cell fates have been reported from different laboratories, a consensus emerged that neural differentiation from PSCs toward the cortical (or anterior) fate is the default. To obtain other neural fates, regionalized factors (or patterning signals) are indispensable. It has been reported that long-term expanded human ESC-derived neural stem cells undergo posteriorization with progressive loss of anterior markers from early to later passages, suggesting posteriorization of an initially anterior regional phenotype appears to be a common property for both ESC- and iPSC-derived neural stem cell lines.
have listed some of the factors that were used to generate various types of neurons from different brain regions in Table 1.

Intriguingly, the listed studies show that different laboratories used different protocols with or without pattern signaling to generate the same type of neurons, for example, glutamatergic neurons. This suggests that the derived glutamatergic neurons might reflect their origins in different regions of the brain. Do they possess different characteristics or activities? It remains to be elucidated. Moreover, many laboratories adopted different paradigms to promote the maturity of neurons, in which BNDF was one of the most commonly used neurotrophic factors. It should be cautioned that maturing neurons by supplementing neurotrophic factors might obscure bona fide pathophenotypes. For example, BNDF has been suggested to be one of causative factors in schizophrenia. Therefore, if iPSC-derived neurons supplemented with BNDF for modeling schizophrenia are used, undoubtedly inadvertent consequences will ensue. For this reason, to pursue the investigation of schizophrenia, we set up a protocol for neuron derivation from hPSCs in the absence of neurotrophic factors.16 We were able to detect electrophysiological activities at around 70–80 d of differentiation and a few neurons showed action potentials. Over 60% of derived neurons exhibited action potentials over 120-day differentiation. However, the number of neurons dwindled as differentiation proceeded, which was accompanied with an increase in the number of astroglial cells. The cause of neuron loss might be attributed to detaching from

Table 1. A summary of protocols used to derive neurons from human pluripotent stem cells.

| TYPE OF NEURONS | COATING | CULTURING | PROTOCOL FOR NEURAL DIFFERENTIATION | NEURAL MATURATION | REF. |
|-----------------|---------|-----------|-------------------------------------|------------------|-----|
| Cortical neurons | Non-adherent | 3-D spheres | KSR/NIM + LDN193189 + SB431542 + XAV939 | NB medium + BDNF + GDNF | 37 |
| Glutamatergic neurons | Matrigel | Monolayer | PSC/NIM + LDN193189 + SB432542 + cyclopamine + FGF2 | NIM + BDNF | 46 |
| | Laminin or FBS | EB + attachment | NIM + Dorosomorphin + SB431542 | NDM + cAMP + IGF + BDNF + GDNF | 5 |
| | Laminin or PLO | EB + attachment | Basic neural medium free of morphogens | Basic neural medium free of morphogens | 49 |
| GABAergic neurons | Matrigel | Monolayer | N2B27 + SB431542 | N2B27 | 16, 29 |
| | Laminin or PLO | EB + attachment and neural stem cell lines | | Co-culturing with mouse astrocytes | |
| | Laminin or PLO | EB + attachment | NIM + SHH or purmorphamine | NB medium + VPA, BDNF, GDNF, IGF, AA, cAMP | 26 |
| Dopaminergic neurons | Matrigel | Neurospheres | NMM + SB431542 + dorosomorphin | NMM + SHH, FGF8 + BDNF + GDNF + cAMP + AA | 4 |
| | Laminin or PLO | EB + attachment | Basic neural medium free of morphogens | Basic neural medium + FGF8 + SHH | 49 |
| | CellStart | EB + attachment | NIM + FGF2 | NB medium + SHH + FGF8 + BDNF + GDNF + TGF-β3 + AA + cAMP | 42, 43 |
| | hESC coculture on hMSS-Wnt cells | Monolayer | SRM/N2 + noggin | N2 + SHH + FGF8 + BDNF + AA | 40 |
| Cholinergic neurons | Matrigel | Neurospheres | NMM + SB431542 + dorosomorphin | In NMM from day 15 to day 27 | 4 |
| | Non-adherent | EB | CDM + SB431542 | NEM + SB431542 + FGF2 + EGF + heparin | 8 |
| Cerebellar Purkinje neurons | Matrigel | Neurospheres | NMM + SB431542 + dorosomorphin | In NMM from day 15 to day 45 | 4 |
| Motor neurons | Non-adherent | 3-D spheres | KSR/NIM LDN193189 + SB431542 + RA + SHH + BDNF | NB media + BDNF + GDNF + CNTF | 37 |
| | Laminin or PLO | EB + attachment | Basic neural medium free of morphogens | Basic neural medium + RA + SHH | 49 |

KSR (KnockOut™ Serum Replacement medium); NIM (neural induction medium); LDN193189 (BMP inhibitor); SB431542 (activin/TGF-binhibitor); XAV939 (Wnt signaling inhibitor); NB (NeuroBasal medium); BDNF (brain-derived neurotrophic factor); GDNF (glial cell-derived neurotrophic factor); PSC (Pluripotent Stem Cell medium); Cyclopamine (Hedgehog signaling pathway inhibitor); PLO (poly-L-ornithine); FGF2 (fibroblast growth factor 2); EB (embryoid body); FBS (fetal bovine serum); dorosomorphin (BMP inhibitor); NDM (neural differentiation medium); cAMP (cyclic AMP); IGF (insulin growth factor); N2B27 (N2B27 medium); PSC (Pluripotent Stem Cell medium); SHH (Sonic hedgehog); purmorphamine (Sonic hedgehog agonist); VPA (valproic acid); AA (ascorbic acid); NMM (neuronal maintenance medium); FGF8 (fibroblast growth factor 8); TGF-β3 (Transforming Growth Factor-β3); hESC (human Embryonic Stem Cells); hMSS-Wnt (mouse stromal cell line MSS-Wnt1); SRM (Serum Replacement Medium); N2 (N2 medium); noggin (BMP inhibitor); CDM (chemically defined media); NEM (Neural Expansion Media); RA (retinoic acid)
dishes or dying out due to long-term culturing. In general, we found that the matrigel-coated surface produced more astroglial cells than the laminin-coated surface. Nevertheless, the former rendered a better cell adhesion.

It is well received that 3-D systems are advantageous over 2-D systems in terms of mimicking in-vivo microenvironments. We would like to point out an interesting phenomenon during hPSC-based neural differentiation. We often observe the formation of 3-D aggregates in our 2-D monolayer culturing systems (matrigel- or laminin-coated surfaces) during the process of neural differentiation either from hPSCs, hNSCs, or neural rosettes (Fig. 1). These 3-D aggregates have a capacity to form spheres. We postulate that the more primitive cells have a higher capacity for forming 3-D aggregates, which might reflect their characteristics in vivo. Our study suggests the end cells derived from 2-D systems are not less useful than those from 3-D systems regarding the recapitulation of neural development. Moreover, our system

![Figure 1](image)

**Figure 1.** A 3-D aggregate captured during neuronal differentiation from human neural rosettes using a 2-D system. This suggests that cells interact within a 3-D environment during a 2-D culturing system. Human ESCs were plated onto Matrigel-coated plates at 1000 cells/cm², and changed to N2B27 medium supplemented with 20 μM SB431542 next day, which was then replaced with fresh N2B27 medium every other day. Neural rosettes, which formed around 9-day differentiation, were harvested and transferred onto Matrigel or Laminin-coated plates. Aggregates are normally formed 2 weeks after transfer. Scale: 10X.

### Table 2. A summary of studies in brain-related diseases using 3-D systems.

| Name of 3-D MODEL | SOURCE | COATING | CULTURING | MODELING | OUTCOME | REF. |
|-------------------|--------|---------|-----------|----------|---------|------|
| Forebrain-specific organoids 3-D neuro-spheroids | hiPSCs | Matrigel | Spinning bioreactor | Zika virus infection and microcephaly | Zika virus causes increased cell death and reduced proliferation | 36 |
| | hiPSCs | Hydrogel | Hydrogel | Alzheimer disease | BACE1 and γ-secretase inhibitors showed less potency in decreasing Aβ levels | 23 |
| Cerebral organoids | hiPSCs hESCs | Matrigel | Spinning bioreactor | Fetal neocortex | Gene expression programs remarkably similar to those of the fetal tissue | 6 |
| | hiPSCs hESCs | Matrigel | Spinning bioreactor | Microcephaly | Progenitor zones in patient-derived tissues display premature neural differentiation at the expense of early progenitor | 21 |
| | hESC | Non | Flask bioreactors | Zika virus infection and microcephaly | Zika virus activates Toll-like receptor 3, which triggers apoptosis and attenuates neurogenesis | 11 |
| Cortical spheroid | hiPSCs | Non | Non-adherent conditions in the absence of extracellular scaffolding | Pyramidal neurons | Electrophysiologically mature, displaying spontaneous activity, and formation of functional synapses | 34 |
| 3-D neural aggregates | hmNPC | poly-L-ornithin-fibronectin | Stirred culture systems with orbital shaking | The imaging of human differentiated 3-D neural aggregates | 3-D CNS cell model can be enhanced by imaging techniques | 17 |
| 3-D multicellular spheroids system | hpAs hpBECs hpPs | Non | Hanging droplet culture plates | The blood brain barrier | The complex interplay of endothelial cells, pericytes, and astrocytes in the cerebral microvasculature | 45 |
| Telencephalic organoid | hiPSCs | Non | Free-floating tridimensional (3-D) culture method | Autism spectrum disorders | Overproduction of GABAergic inhibitory interneurons caused by increased FOX1 gene expression | 28 |
| Brain organoids | hiPSCs | Matrigel | Spinner flask | Zika virus infection | Infected organoids were 40% smaller compared with controls after 11 days | 15 |
| | hiPSCs chiPSCs | Matrigel | Spinning bioreactor | Zika virus in birth defects | A significant decrease in the number of PAX6-expressing neural progenitor cells and differentiated neurons | 9 |
| Midbrain organoids | hiPSCs hESC | Non | Orbital shaker | Midbrain tissues | A long-lived nervous tissue containing mature dopaminergic neurons | 44 |

**Table 2** - A summary of studies in brain-related diseases using 3-D systems.

- **hESCs** - human Embryonic Stem Cells; **hiPSCs** - human induced Pluripotent Stem Cells; **hmNPC** - human midbrain-derived Neural Progenitor Cells; **hpAs** - human cerebral Astrocytes; **hpBECs** - primary human Brain microvascular; **Endothelial Cells** hpPs - human brain vascular Pericytes; **chiPSCs** - chimpanzee induced pluripotent Stem Cells.
can, more or less, achieve what the 3-D system can do and the derived cells can be examined straight away without further processing (e.g., frozen section).\textsuperscript{16} Additionally, the neural development occurred naturally in our system without interference by BDNF and NT3 needed in some 3-D systems, which might mask the phenotypes of neuro-development. Nevertheless, it is not our intention to promote 2-D systems over 3-D systems. The advance of 3-D culturing has benefited the studies in brain-related diseases, which are shown in Table 2.

In conclusion, although perfect human disease models are beyond our reach, the hPSC-based system is one valid alternative. However, it is still a long way for hPSCs to generate perfect human disease models, especially for brain-related diseases, perhaps the advance of 3-D systems might bring it one step closer.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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