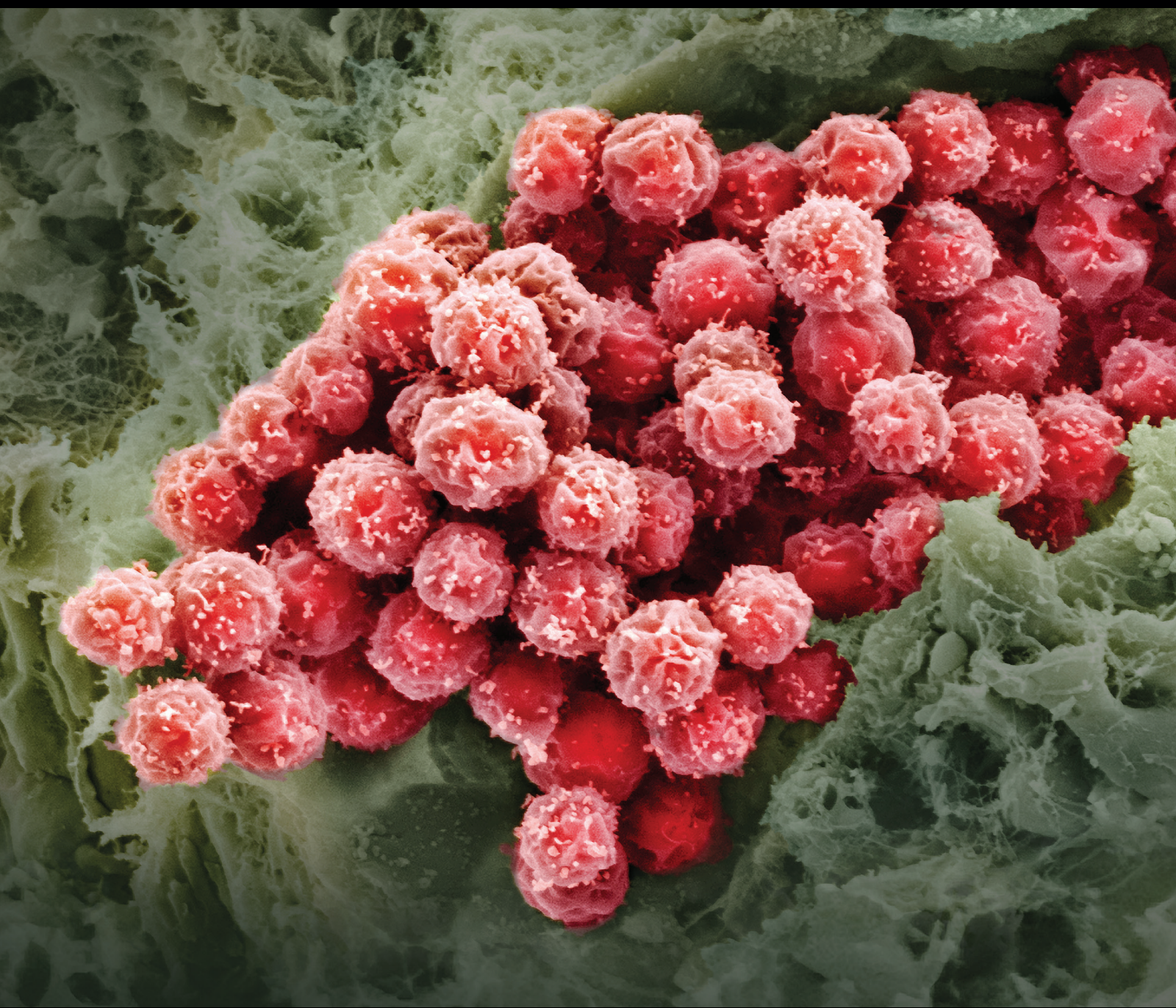
Stem Cells International

Modeling CNS Development and Disease

Guest Editors: Jason P. Weick, Jason S. Meyer, Julia Ladewig, Weixiang Guo, and Yan Liu



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Editorial Board

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Editorial

Modeling CNS Development and Disease

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The ultimate goals for stem cell researchers are uncovering the fundamental mechanisms of cellular biology as well as developing treatments for disease and/or injury. The central nervous system (CNS) presents an unparalleled challenge for these studies due to the diversity of cell types and their interrelated functions in health and disease. Furthermore, the endpoints for determining phenotypic maturation are complicated by the fact that cells, especially neurons, express a plethora of receptors and ion channels that control excitability and plasticity which vary greatly between CNS regions. For this reason, extensive effort has been placed on developing methods to differentiate appropriate populations of cells that reside within the CNS (e.g., neuronal subtypes, astrocytes, and oligodendrocytes). These studies typically employ phenotypic validation by the use of expression analysis for various protein markers of regionalized populations, with a burgeoning effort to verify functional outcomes. Much of this work has been informed by basic developmental studies that have identified cell-intrinsic and cell-extrinsic signaling mechanisms, as well as transcriptional programs involved with cell fate specification.

While early work focused on the use of mesenchymal stem cells, embryonic carcinoma cells, and fetal stem cells, more recent efforts have focused on the areas of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). These sources typically require slightly different methods to direct differentiation to various CNS fates, although many mechanisms appear to converge on common signaling pathways. Many recent studies have even successfully uncovered cell-type specific phenotypes that are appropriate for various human diseases. However, despite these advances, limitations still hamper the application of human stem cells for CNS development/disease studies. First, most differentiation protocols lead to a heterogeneity of cells in terms of both their "regional" markers and their maturational states. Furthermore, the number and diversity of available fate-specific biomarkers remain insufficient to unambiguously identify regional and transmitter phenotypes.

In this special issue, we highlight both the advancements and challenges of directed differentiation of various neuronal and glial cells from pluripotent stem cells. We hope this collection of works will provide readers with a resource to understand directed differentiation, functional maturation, and transplantation-induced changes to host environments.

The article by P. Prajumwongs et al. reviews the current state of knowledge regarding neural induction of human pluripotent stem cells, detailing the signaling pathways and timing of critical events such as neuroepithelial differentiation. Emphasis is placed on the comparison between *in vitro* and *in vivo* development and the utility of hESCs to model human-specific signaling processes. The article concludes with a series of brief descriptions about various developmental and neurodegenerative diseases and how iPSCs have been used to model their underlying molecular and cellular pathologies.

The review article from A. Zirra et al. gives a detailed overview of the development of the neural tube and how these *in vivo* principles provided the basis for *in vitro* neural induction strategies of human pluripotent stem cells (hPSCs). The article highlights the basic stages of neural development including neural induction, neurulation, and neural patterning. Here they focus on signaling pathways, morphogenes, and patterning factors described to be crucial for the development of distinct brain regions. The authors then discuss how knowledge from *in vivo* brain development has been translated by many groups to direct the differentiation of hPSCs to clinically relevant region-specific neurons *in vitro*. Finally, they give an outlook of how human brain development data can be utilized for improving directed differentiation protocols and validating the thereof derived region-specific subpopulations of human neurons.

The article by F. Cavaliere et al. reviews the use of organotypic hippocampal slice cultures as a model for understanding neurogenesis under normal and pathological conditions. They first present a historical summary of the development of slice cultures from neonatal, and more recently adult animals, for the study of neurogenesis from both SVZ and DGZ. The article also describes results from pathological studies of slice cultures in the context of ischemia, Parkinson's, and Alzhemier's disease, as well as regeneration of neurons in the spinal cord. The authors suggest that the maintenance of the 3D architecture and cellular composition of these cultures make them unique among *in vitro* models for these types of studies.

The primary research article from T. H. Shin et al. tested the effects of mesenchymal stem cells on polyamine levels in both *in vitro* and *in vivo* models of ischemic stroke. Polyamines are low molecular weight compounds secreted in high concentrations from the brain and are important indicators of metabolic function, especially in response to injury or disease. The authors found that middle cerebral artery occlusion (MCAo) *in vivo* and oxygen-glucose deprivation (OGD) of a neural cell line *in vitro* significantly altered a plurality of polyamine levels. Importantly, the authors found that disruptions of polyamine levels by MCAo- and OGD-induced ischemic conditions could be restored to near-normal levels after introduction of human bone marrow-derived mesenchymal stem cells.

Finally, the review article from J. P. Weick highlights the efforts by many groups to understand the functional phenotypes of pluripotent stem cell-derived neurons as well as directly converted/induced neurons (iNs). This article focuses on the assessment of developmental maturation of forebrain neurons via analysis of forebrain glutamatergic and GABAergic currents, comparing these *in vitro* findings to *in vivo* data from rodent models. The author then discusses recent results modeling various neurological disorders using iPSC-derived neurons and iNs. He concludes by critically reflecting on the use of these two cell types for developmental and disease studies and provides suggestions on how to optimally determine and report neuronal function.

Together, the articles in this special issue highlight recent advances in our understanding of the directed differentiation and functional properties of stem cells and describe several new approaches to model and develop novel therapies for neurological disease and injury.

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Research Article

Restoration of Polyamine Metabolic Patterns in *In Vivo* and *In Vitro* Model of Ischemic Stroke following Human Mesenchymal Stem Cell Treatment

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We investigated changes in PA levels by the treatment of human bone-marrow-derived mesenchymal stem cells (hBM-MSCs) in ischemic stroke in rat brain model and in cultured neuronal SH-SY5Y cells exposed to oxygen-glucose deprivation (OGD). In ischemic rat model, transient middle cerebral artery occlusion (MCAo) was performed for 2 h, followed by intravenous transplantation of hBM-MSCs or phosphate-buffered saline (PBS) the day following MCAo. Metabolic profiling analysis of PAs was examined in brains from three groups: control rats, PBS-treated MCAo rats (MCAo), and hBM-MSCs-treated MCAo rats (MCAo + hBM-MSCs). In ischemic cell model, SH-SY5Y cells were exposed to OGD for 24 h, treated with hBM-MSCs (OGD + hBM-MSCs) prior to continued aerobic incubation, and then samples were collected after coculture for 72 h. In the *in vivo* MCAo ischemic model, levels of some PAs in brain samples of the MCAo and MCAo + hBM-MSCs groups were significantly different from those of the control group. In particular, putrescine, cadaverine, and spermidine in brain tissues of the MCAo + hBM-MSCs group were significantly reduced in comparison to those in the MCAo group. In the *in vitro* OGD system, N^1-acetylspermidine, spermidine, N^1-acetylspermine, and spermine in cells of the OGD + hBM-MSCs group were significantly reduced compared to those of OGD group.

1. Introduction

Bone-marrow-derived mesenchymal stem cells (BM-MSCs) are regarded as promising agents in ischemic stroke therapy [1–4] because of their differentiation plasticity, easy attainability, and weak immune response inducing ability [5–8]. In addition, mesenchymal stem cells are also known as suppressor of inflammation that is related to pathology of stroke [9, 10]. Accordingly, BM-MSCs have proven to be effective in ameliorating functional deficits as well as restoring tissues damages that occur after stroke [11, 12].

In previous clinical report, autologous human bone-marrow-derived mesenchymal stem cell (hBM-MSC) transplantation in patients with ischemic stroke suggested hBM-MSC's potential for providing functional recovery [13]. Although this study suggests that autologous hBM-MSC transplantation may be a safe treatment method for ischemic stroke, the precise underlying therapeutic mechanisms of

hBM-MSC transplantation remain unknown. Furthermore, complex chemical, cellular, and physiological processes are involved in the dynamic regulation of metabolites during diseased states. Therefore, it is extremely difficult to instantaneously monitor and profile the dynamic metabolic changes in diseased state and therapeutic outcomes accurately. Among the diverse biogenic compounds with low-molecular weights occurring in metabolic pathways, polyamines (PAs) serve as the most important biochemical indicator for various pathological conditions [14–16]. The naturally occurring di-, tri-, and tetra-amines, polyamines (putrescine, cadaverine, spermidine, and spermine), which occur in metabolic pathways, are detected in high concentrations in the brain [17, 18]. PAs are secreted from intracellular compartments in several central nervous system (CNS) injuries, including focal cerebral ischemia in the ischemic cascade [18, 19]. Unregulated catabolism of PAs induces the production of harmful metabolites, such as hydrogen peroxide, by chelating Fe^{2+} via Fenton's reaction [20], thus increasing ischemic injury [21–23]. However, the precise characterization and the explicit role of PAs in brain ischemic conditions remain unknown.

In previous reports, spermine and spermidine were reported as free radical scavengers in rat brain homogenates, capable of reducing lipid peroxidation induced by prooxidant agents, including quinolinic acid (QA), iron (Fe^{+2}), and sodium nitroprusside (SNP) [17, 24]. In particular, spermine reduced infarction and neurological deficit in a rat ischemic model as determined by using magnetic resonance imaging [25]. Even though the blood-brain barrier (BBB) strictly regulates the exchanges between brain and blood in normal metabolic conditions, induction of polyamine putrescine in cold-injury brains changes the integrity of focal BBB dysfunction [26]. These studies, taken together, support the suggestion that PAs and their metabolism play an important role as critical metabolomic components associated with cellular and chemical events during neurodegeneration following cerebral ischemia.

In order to further elucidate the beneficial or harmful role of altered PA levels as oxidant modulating agents in stroke, the present study was designed to analyse PA changes by evaluating treatment effects of hBM-MSCs in two different ischemic models. The first model utilized ischemic stroke condition in rats that had undergone middle cerebral artery occlusion (MCAo), while the second model employed oxygen-glucose deprivation (OGD) in cultured neuronal SH-SY5Y cells. In our previous report, regarding the rat stroke model, although there was no significant difference in infract volume and neurological status in MCAo induced groups irrespective of hBM-MSCs treatment before 7 days [27], the levels of free fatty acids (FFAs) in brain samples varied significantly in MCAo and hBM-MSC-transplanted MCAo groups in comparison to the control group at 5 days after MCAo. The study found that myristic, linoleic, and eicosenoic acids' levels of the hBM-MSC-transplanted MCAo group were significantly less than those of the control group [28]. Thus, we analysed polyamine metabolites at 5 days as this reflects the phenotype relatively well. In light of these observations, other metabolomic profiles, such as those related to PAs, may be linked with physiological, chemical, or cellular conditions in hBM-MSC transplantation in ischemic condition. Therefore, we extended our study to evaluate the metabolic patterns of PAs using both *in vivo* animal and *in vitro* cell models.

Although the therapeutic effects of stem cells were studied in ischemic animal models, the analysis of changes in PAs profiling associated with these conditions has not yet been attempted. Therefore, in the present study, simultaneous metabolic profiling analysis of aliphatic and acetylated PAs was performed to examine altered metabolic patterns of PAs in MCAo rat brains following intravenous hBM-MSCs injection and OGD SH-SY5Y cells treated with hBM-MSCs using coculture system. Outcomes from present experiments may provide new insight into our understanding of the complexity of biochemical and physiological events that occur in ischemic brain injury and after hBM-MSC replacement therapy in treatment for the stroke and other related disorders.

2. Results

2.1. Transplanted hBM-MSCs Were Detected in the Ischemic Border Zone. To assess the MCAo model, 2,3,5-triphenyltetrazolium chloride (TTC) staining was performed with ischemic rats. Infarcts, appearing as white areas, were mostly located within the ipsilateral cortex and striatum (Figure 1(a)). To trace transplanted hBM-MSCs *in vivo*, we assessed the fluorescence of cells labelled with PKH-26 in ischemic brain regions 4 days after injection. The ischemic region was identified by DAPI and hBM-MSCs labelled with NuMA were found in the margin (about 3–5% of 1×10^6 hBM-MSCs, Figure 1(b)); these results are consistent with a previous report in the same model [27, 28]. However, we did not find NuMA and PKH-26 positive cells in the brain sections of control group and MCAo group. After confirmation of the stroke animal model and transplantation of hBM-MSCs in the ischemic brain region, we analysed the metabolic patterns of PAs four days after the transplantation of hBM-MSCs into the MCAo model.

2.2. PAs Levels in the Brain of Rat Model. To investigate the change of PA levels between control, control + hBM-MSCs, MCAo, and MCAo + hBM-MSCs (1×10^6) groups, brain PA profiling analysis was performed. Nine PAs were detected in rat brain tissue with great variations in levels being observed within each group (Table 1). Compared to the control, the levels of putrescine, cadaverine, N^1-acetylspermidine, and spermidine were significantly increased in the MCAo group, while the levels of putrescine and spermidine were significantly increased in the MCAo + hBM-MSCs group. However, there was no significant difference in polyamine levels between control and control + hBM-MSCs, except for spermidine increment. In addition, putrescine, cadaverine, and spermidine were significantly reduced in the MCAo + hBM-MSCs group in comparison to the MCAo group, but still they were of higher levels compared to control group.

Table 1: Polyamine levels in brain tissues from rats in the control group, group with transient MCAo, and the MCAo group following transplantation with hBM-MSCs.

Number	Compound	Polyamine levels (nmol, mean ± SD) in rat brain tissue (g)				
		Control group ($n = 10$)	Control + hBM-MSCs group ($n = 7$)	MCAo group ($n = 7$)	MCAo + hBM-MSCs group ($n = 7$)	p value[d]
1	N^1-Acetylputrescine	374.7 ± 15.3	365.6 ± 12.0 (0.1)[a]	382.3 ± 22.8 (0.2)[b]	376.6 ± 15.0 (0.4)[c]	0.3
2	N^1-Acetylcadaverine	419.2 ± 17.7	408.3 ± 13.8 (0.1)	428.1 ± 26.9 (0.2)	421.1 ± 14.7 (0.4)	0.3
3	Putrescine	109.3 ± 5.0	107.4 ± 3.6 (0.2)	141.9 ± 14.2 (0.000003)	129.7 ± 13.6 (0.0003)	0.06
4	Cadaverine	59.5 ± 5.3	64.9 ± 11.9 (0.1)	73.7 ± 9.8 (0.0008)	62.4 ± 5.2 (0.1)	0.01
5	N^1-Acetylspermidine	137.0 ± 9.9	145.1 ± 19.3 (0.1)	176.6 ± 24.4 (0.0002)	158.0 ± 25.7 (0.02)	0.1
6	N^8-Acetylspermidine	231.8 ± 10.4	225.6 ± 6.9 (0.1)	244.2 ± 14.6 (0.03)	235.5 ± 9.3 (0.2)	0.1
7	Spermidine	172.6 ± 13.8	201.4 ± 13.4 (0.0003)	248.4 ± 44.3 (0.00006)	208.6 ± 37.6 (0.007)	0.05
8	N^1-Acetylspermine	140.7 ± 24.2	138.2 ± 28.8 (0.4)	138.4 ± 20.5 (0.4)	130.7 ± 21.2 (0.2)	0.3
9	Spermine	513.6 ± 21.3	503.5 ± 19.4 (0.2)	520.7 ± 30.4 (0.3)	513.6 ± 18.4 (0.5)	0.3

[a]Student's t-test at 95% confidence level on the mean values of control and control + hBM-MSCs groups.
[b]Student's t-test at 95% confidence level on the mean values of control and MCAo groups.
[c]Student's t-test at 95% confidence level on the mean values of control and MCAo + hBM-MSCs groups.
[d]Student's t-test at 95% confidence level on the mean values of MCAo and MCAo + hBM-MSCs groups.

Table 2: Polyamine levels in cells from control, OGD, and OGD + hBM-MSCs groups for 72 h.

Number	Compound	Polyamine levels (ng, mean ± SD) in cells (4×10^5)			
		Control group ($n = 3$)	OGD group ($n = 3$)	OGD + hBM-MSCs group ($n = 3$)	p value[c]
1	N^1-Acetylputrescine	ND[d]	ND	ND	
2	N^1-Acetylcadaverine	ND	ND	ND	
3	Putrescine	16.4 ± 1.5	21.8 ± 0.9 (0.002)[a]	28.1 ± 0.9 (0.0003)[b]	0.001
4	Cadaverine	12.5 ± 0.2	12.4 ± 0.4 (0.794)	12.6 ± 0.3 (0.861)	0.501
5	N^1-Acetylspermidine	45.6 ± 1.8	66.5 ± 0.8 (0.0003)	54.3 ± 4.7 (0.026)	0.006
6	N^8-Acetylspermidine	64.4 ± 0.6	56.7 ± 4.0 (0.047)	50.1 ± 3.3 (0.03)	0.082
7	Spermidine	132.5 ± 3.0	212.1 ± 16.7 (0.0002)	180.4 ± 6.9 (0.004)	0.024
8	N^1-Acetylspermine	63.4 ± 6.0	127.3 ± 9.7 (0.0005)	110.6 ± 0.6 (0.0005)	0.050
9	Spermine	64.9 ± 6.2	243.8 ± 15.1 (0.0002)	177.6 ± 9.5 (0.0003)	0.001

[a]One-way ANOVA, p value < 0.05 between control and OGD groups.
[b]One-way ANOVA, p value < 0.05 between control and OGD + hBM-MSCs groups.
[c]One-way ANOVA, p value < 0.05 between OGD and OGD + hBM-MSCs groups.
[d]Not determined.

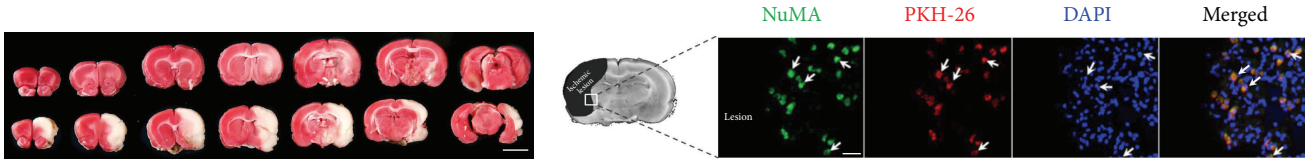

Figure 1: 2,3,5-Triphenyltetrazolium chloride (TTC) staining and hBM-MSC staining with anti-NuMA antibody, PKH-26, and DAPI. (a) TTC-stained coronal brain sections of control (upper panel) and 5 days after MCAo (bottom panel). Scale bar = 5 mm. (b) hBM-MSCs labelled 5 days after MCAo. The localization of hBM-MSCs in the ischemic region was identified by NuMA (green), PKH-26 (red), and DAPI (blue); white arrows indicate double-positive cell. Scale bar = 20 μm.

The levels of each of the nine PAs in brain tissues of the MCAo and MCAo + hBM-MSCs groups were normalized to the corresponding mean values from the control group (Figure 2). PA levels of the MCAo and MCAo + hBM-MSCs groups display multiple control mean values ranging from 0.9 to 1.4. Compared to the control group, four and three PAs were observed as having significant variation in the MCAo and MCAo + hBM-MSCs groups, respectively. In both the MCAo and MCAo + hBM-MSCs groups, the level of spermidine was found to be the most altered, followed by putrescine in comparison to the control group. Interestingly, the PAs, which were elevated in MCAo, were reduced in the MCAo + hBM-MSCs group, with the level of most PAs approximating the level of the control group. Representative SIM chromatograms also revealed that putrescine, cadaverine, and spermidine of the MCAo + hBM-MSCs group were significantly lower than those of the MCAo group, but still they were of higher levels compared to control group (Figure 3).

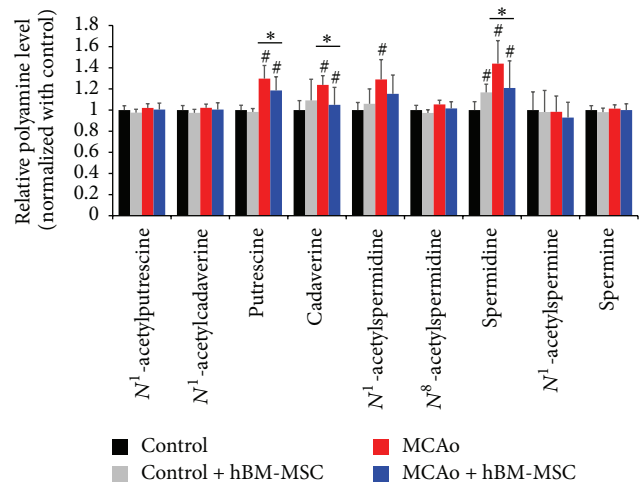

Figure 2: Bar plot of MCAo and MCAo + hBM-MSCs groups based on mean levels of the nine polyamines in rat brain tissues as variables after normalization to the corresponding mean values of the control group. $^{\#}p < 0.05$ (comparison with control) and $^{*}p < 0.05$ (comparison between MCAo and MCAo + hBM-MSCs groups). Data shown are means ± SE for three independent experiments.

2.3. PAs Levels in Cells under OGD. To mimic the MCAo animal model, we used Boyden chamber (Transwell) to assess whether coculture with hBM-MSCs exhibited the same effects to OGD SH-SY5Y system as an *in vitro* ischemic model. PA profiling analysis was performed with the control ($n = 3$), OGD ($n = 3$), and OGD + hBM-MSCs ($n = 3$) groups. Seven PAs were detected in the cells of each group (Table 2). The levels of putrescine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine, N^1-acetylspermine, and spermine were significantly elevated in the OGD

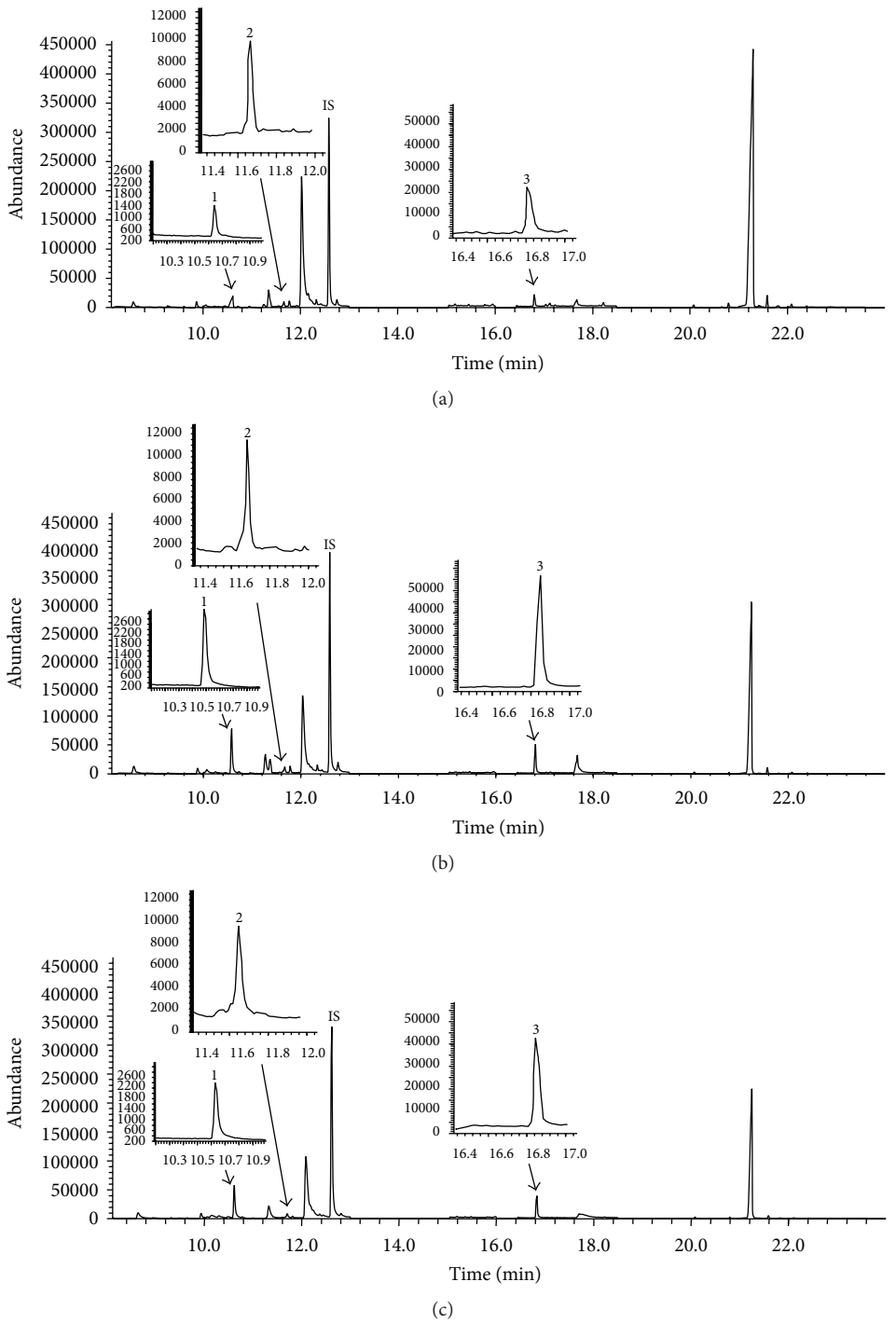

Figure 3: GC-SIM-MS chromatograms of putrescine (1), cadaverine (2), and spermidine (3) in rat brain tissues from control (a), MCAo (b), and MCAo + hBM-MSCs groups (c). IS: internal standard (1,6-diaminohexane).

group compared to the control group. In particular, N^1-acetylspermine and spermine were increased by more than 2-fold. Compared to the control group, the levels of putrescine, N^1-acetylspermidine, spermidine, N^1-acetylspermine, and spermine in the OGD + hBM-MSCs group were significantly increased, whereas N^8-acetylspermine was significantly reduced. Compared to the OGD group, the level of putrescine was significantly increased in the OGD + hBM-MSCs group, whereas the levels of N^1-acetylspermidine, spermidine, N^1-acetylspermine, and spermine were significantly decreased.

Similar to the procedures used in the MCAo animal model, each level of the seven PAs in cells of the OGD and OGD + hBM-MSCs groups was normalized to the corresponding mean values from the control group. These

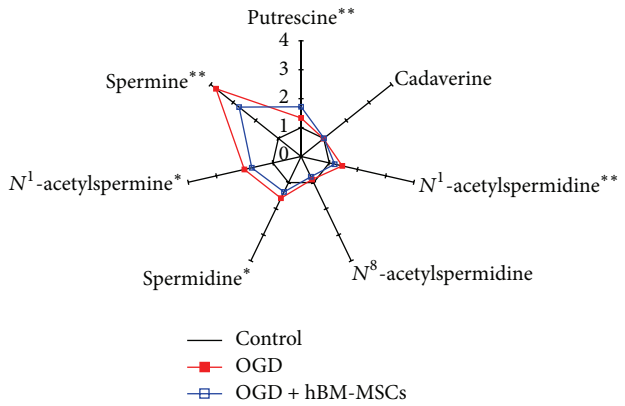

Figure 4: Star symbol plots of OGD and OGD + hBM-MSCs groups based on the mean levels of the seven polyamines in cultured cells for 72 h as the variables after normalization to the corresponding control group mean values. $^{*}p < 0.05$ and $^{**}p < 0.01$ (comparison between OGD and OGD + hBM-MSCs groups). Data shown are means ± SE for three independent experiments.

normalized values were then utilized for star graphs composed of seven rays, so that the differences in the mean values among the control, OGD, and OGD + hBM-MSCs groups were exhibited clearly (Figure 4). PA levels from the OGD and OGD + hBM-MSCs groups were elevated manifold (ranging from 0.8 to 3.8), compared to the control mean values. In the OGD and OGD + hBM-MSCs groups, spermine was the most changed followed by N^1-acetylspermine, compared to the control group. Interestingly, the PAs, which were elevated in OGD, except for putrescine, were reduced in the OGD + hBM-MSCs group, similar to the *in vivo* results. The representative SIM chromatograms also revealed that putrescine and spermine of the OGD + hBM-MSCs group were considerably altered compared to the OGD group (Figure 5).

3. Discussion

To the best of our knowledge, the present study is the first demonstration of altered PA metabolism in the brains of ischemic rats and OGD cells following transplantation of hBM-MSCs. Despite the complexity of the generated results, the present findings provided evidence that hBM-MSCs transplantation could ameliorate PA metabolic dysfunctions associated with ischemic cellular injuries in both brain tissues and OGD cells.

In the ischemic stroke rat model, compared to the control group, the levels of 4 of 9 PAs were increased in brains of rats in the MCAo group. All PAs that were increased in the MCAo group, except for N^1-acetylspermidine, were considerably reduced in the MCAo + hBM-MSCs group (Table 1). In the OGD cell model, the levels of 5 of 7 PAs, except for cadaverine and N^8-acetylspermine, were increased in oxygen-glucose deprived cells compared to the control group. However, all elevated PAs in the OGD group, except for putrescine, were noticeably reduced in the OGD + hBM-MSCs group (Table 2). These results collectively suggested that the disturbance of PA metabolism by MCAo- and OGD-induced ischemic condition could be restored to a near-normal state after transplantation of hBM-MSCs.

In the present study, hBM-MSCs transplantation showed limited restoration of cellular polyamine homeostasis in rat MCAo models because complete restoration to basal level depends on various internal and external factors. Moreover, polyamine metabolism is a reversible process in which various polyamines alter between intermediate forms [29], which makes quantification of polyamines in cells difficult. This also contributes to limited interpretation of our data regarding polyamine alterations in the OGD model. In order to accurately quantify polyamines in OGD model, tracing of polyamines by radiolabelling their precursor ornithine will be required.

In the ischemic animal model, changes in the metabolite profile of spermidine were observed in the MCAo and MCAo + hBM-MSCs groups. This supported the possibility that MCAo might induce the elevation of spermidine through the putrescine metabolic pathway, such as by the activation of spermidine synthase (SpdS) due to ischemic conditions [30]. Also, spermine and spermidine concentrations were reported to increase in response to acute hypoxia in fetal rat brains that coincided with cell differentiation and growth, suggesting a protective role in hypoxic brain [31]. Since large physiological changes in putrescine and cadaverine have been described in brain ischemia, abnormally high levels of these metabolites observed in the present MCAo group could contribute to the further imbalance in other PAs [17]. In addition, upregulation of both spermidine and putrescine as well as their acetylated form suggests an increase in biosynthesis as well as in the interconversion pathway. Our results provided evidence that transplantation of hBM-MSCs in ischemic brain was capable of restoring metabolic disturbances caused by ischemia and further that prevention of ischemic stroke might be related, in part, to normalization of the release of PAs from the intracellular compartment in the ischemic brain.

Stem cells are responsible for cell renewal and maintenance of tissue homeostasis [32]. Increasing evidences indicate that hBM-MSCs promote functional recovery in animal model of ischemic stroke. We reported that upregulation of the endogenous recovery mechanism at the peri-infarct area (neurogenesis) has an important role of hBM-MSCs in functional recovery after ischemic stroke after 14 days [27]. In addition, transplantation of hBM-MSCs restored free fatty acid composition in ischemic stroke rat model [28]. In this study, we postulate that transplantation of hBM-MSCs contribute to maintenance of metabolic homeostasis before or during early pathological condition in ischemic stroke rat model.

In a previous study using a similar ischemic MCAo stroke model, both spermine and spermidine levels were reported to be significantly decreased at 6 and 24 h after MCAo in the ischemic cortex compared to the control cortex [33]. On the other hand, current findings showed that the level of spermidine was increased, whereas the level of spermine remained unaltered in MCAo condition when compared to the control condition. These dissimilar results may be attributed, at least in part, to differences in methodological approaches used in preparing brain tissue. We used total brain, obtained from

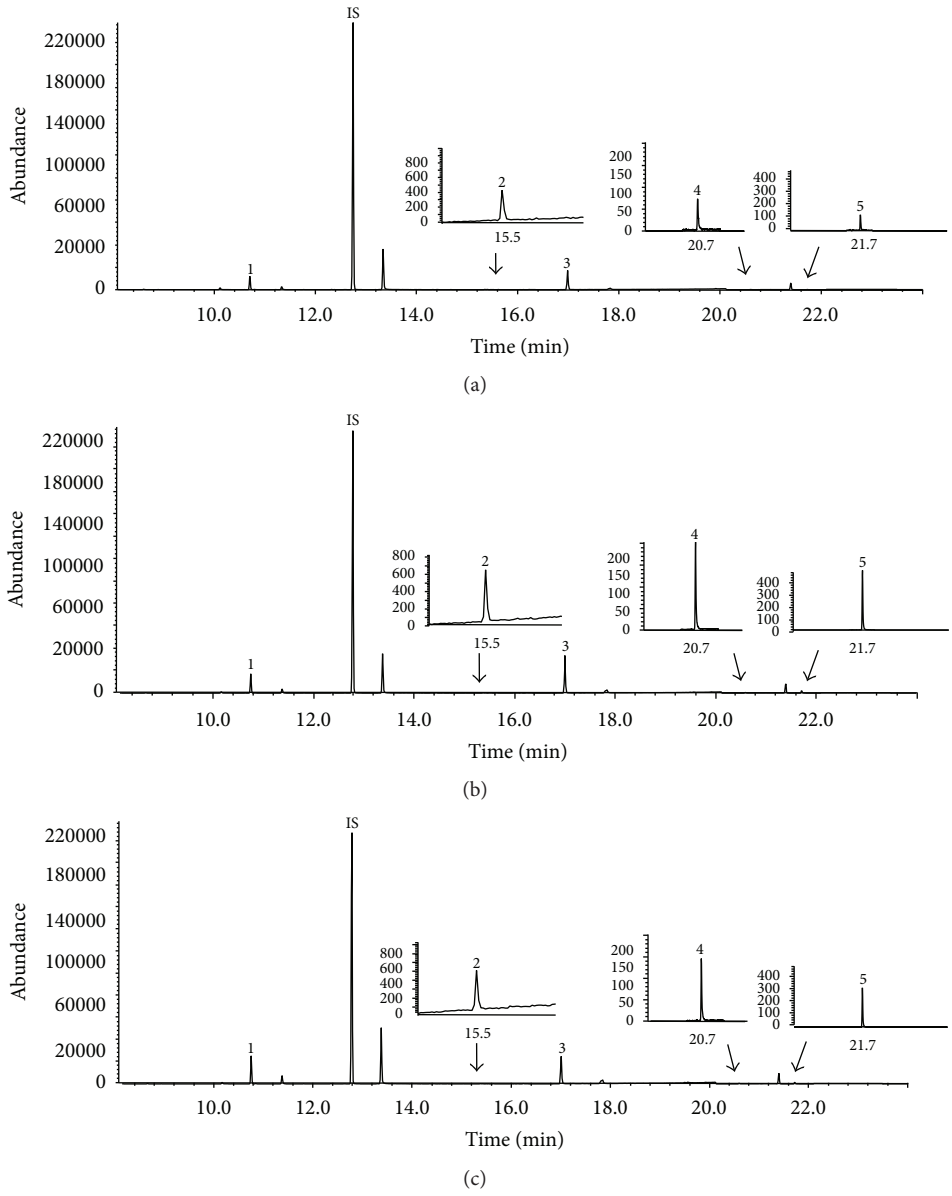

Figure 5: GC-SIM-MS chromatograms of putrescine (1), N^1-acetylspermidine (2), spermidine (3), N^1-acetylspermine (4), and spermine (5) in cells from control (a), OGD (b), and cocultured OGD cells (c) with hBM-MSCs for 72 h. IS: internal standard (1,6-diaminohexane).

brain tissues 5 days after MCAo. Although blood volume contributes only about 1% to total tissue polyamines [34], the animals in the present study were perfused with physiological saline prior to brain tissue collection in order to exclude the possibility of the effect of blood on PA expression. In addition, the spermine in brain tissues of the control group was higher than spermidine, which showed a different pattern in comparison to previous report [35]. This may be explained by our use of the whole brain rather than particular regions of the brain.

In MCAo ischemic rat model, it is important to note that the altered PAs in the ischemic rat brain were measured at only one time point after hBM-MSC transplantation, namely, 4 days after the hBM-MSC transplantation. PA change in brains was also observed at only one time point after ischemia, namely, 5 days after MCAo. Future studies, therefore, should evaluate other postischemic, as well as posttransplant, time points. This may be important for more accurate assessment of the alterations in PA metabolite profiles in a time-dependent manner, allowing the analysis of the impact of ischemia *per se*, as well as of its interaction with hBM-MSC transplantation, as a function of stem cell survival, migration, and functional integration into the host CNS tissue. In addition, other factors, including stem cell dose and its route of delivery, should be investigated in relation to specific biochemical processes involving PA metabolism in order to achieve optimal treatment condition and to validate the potential clinical utility of hBM-MSC stem cell therapy

in ischemic conditions such as stroke and related disease states.

Furthermore, in an OGD coculture system, PA levels were also measured at only one time point, namely, at 72 h after coculture with hBM-MSCs, since, in this case, there were no earlier cytoprotective effects of hMSC. In fact, at 24 h after exposure to OGD, viability of SH-SY5Y cells was about 80% or less in the absence of hBM-MSCs treatment and remained at about the same level after hBM-MSC treatment (data not shown). Therefore, future OGD cell experiments should include analyses using various OGD exposure time and/or varying hBM-MSCs concentrations to better delineate the functional relationship between hBM-MSCs therapeutic effects and altered PA metabolomics in this model. It is also of significant importance to recognize that the present *in vitro* OGD experiments utilized the SH-SY5Y human neuroblastoma cell line, a well-characterized and well-established cell model system for studying neuronal growth *in vitro*. Therapeutic effects of stem cells in ischemic rats, however, are not directly related to neuroblastoma cells but instead are more closely related to other types of cells, such as cortical neurons, microglia, and astrocytes [36, 37]. Future *in vitro* studies concerning the effects of hMSC in ischemic conditions should incorporate the use of these cell lines as well as primary cells which evaluate polyamine effects on receptors (i.e., NMDA) associated with synaptic functions [38]. However, glial cells are immune cells of the brain and assessing the effect of OGD and hBM-MSCs transplantation in these cells and neurons, complex multilevel investigation such as 3D culture is required [39]. hBM-MSCs also induce microgliosis and astrogliosis in transplanted brain [40]. Moreover, secondary paracrine effects of hBM-MSCs on resident microglia and neurons further complicate the hypoxic microenvironment. Such a study will require more careful consideration of several critical factors. Accordingly, the present *in vitro* data and its potential clinical implications should be interpreted cautiously until further work with additional cell lines is completed.

The changes of PA levels from the *in vitro* model are very different from those obtained in ischemic rat model. Particularly, spermine concentration is modified in the presence of OGD and then after hBM-MSCs cocultures. This discrepancy is considered to be responsible for selective neuronal cell line *in vitro* model and presence of several cell types in brain tissues such as neurons, microglia, and astrocytes. Further research is clearly warranted to delineate precise underlying restorative mechanisms of hBM-MSCs transplantation in ischemia, since the involvement of PAs in the ischemic damage and related alterations are still unknown.

4. Materials and Methods

4.1. Transient MCAo Animal Model. Ischemic stroke was introduced by the intraluminal suture occlusion model in a stroke rat model. Adult male Sprague-Dawley rats weighing 250–300 g were anesthetized, by the use of face mask, with 4% isoflurane, and maintained with 1.5% isoflurane in 30% O_2 and 70% N_2O. During the surgery, rectal temperature was maintained at 37.0–37.5°C with heating pads. Transient MCAo using a method of intraluminal vascular occlusion modified in our laboratory was used [28]. Briefly, a 4-0 surgical monofilament nylon suture with rounded tip was introduced through the common carotid artery (CCA) and used to block the lumen of the internal carotid artery (ICA). Two hours after MCAo, reperfusion was performed by withdrawal of the suture until the tip cleared the lumen of CCA. Surgical procedures for sham groups were identical to those used for MCAo surgery, except for omission of the suture insertion. The use of animals was approved by the Animal Care and Use Committee of Ajou University Hospital.

4.2. 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining and Immunohistochemistry. To confirm the establishment of ischemic animal model, rats were anesthetized with chloral hydrate 24 hours after MCAo. Brains were then removed and immediately sectioned coronally into six slices (each 2 mm in thickness) in a rodent brain matrix (Harvard Instrument, South Natick, MA), as described previously [28]. Briefly, brain slices were placed in 2% triphenyltetrazolium chloride (TTC; T-8887; Sigma-Aldrich, St. Louis, MO), incubated at 37°C for 40 min and fixed by immersion in 10% formalin. Using a flatbed color scanner, the stained sections were photographed and scanned for further analysis. The infarcts were mostly located within the ipsilateral cortex and the striatum in three representative slices (Figure 1(a)). For immunohistochemistry, animals were sacrificed one day after MCAo. Brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde. The brains were kept overnight in paraformaldehyde at 4°C and then embedded in a 30% sucrose solution until they sank. Coronal sections 30 μm in thickness were cut using a model CM1800 cryostat (Leica, Wetzlar, Germany). The sections were washed three times with phosphate-buffered saline (PBS), nonspecific binding was blocked with 10% horse serum, and each section was stained with 1 : 500 dilution of mouse monoclonal human nuclei matrix antigen (NuMA; Calbiochem, San Diego, CA). Thereafter, sections were washed and incubated with secondary antibody. To assess the number of hBM-MSCs within the transplant, the total number of NuMA-positive cells in the forebrain (bregma-1, approximately 1 mm) was calculated on ten sequential slides at intervals of 150 μm, and the number of NuMA-positive cells was counted by summing those found on all 10 slides as described previously [27, 28]. To trace transplanted hBM-MSCs *in vivo*, the cells were labelled using a PKH 26 Red Fluorescence Cell Linker Kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, brains were harvested from MCAo or sham-operated animals and fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer. Frozen brains were sectioned with a Cryocut-microtome system (Leica, Germany). Sections were then incubated with 4,6-diamidino-2-phenylindole (DAPI; Fluka, USA) for 15 min at room temperature to counterstain the nuclei and mounted.

4.3. Animals and Experimental Groups. One day after MCAo, animals were randomly divided into three groups as in our previous report [28]: (1) sham ischemia + phosphate-buffered saline (PBS) injection (control, $n = 10$), (2) MCAo + PBS injection ($n = 6$), and (3) MCAo + hBM-MSCs (1×10^6, $n = 7$) injection. All animals were allowed to survive for 5 days after MCAo. MCAo + hBM-MSCs group showed functional recovery with the adhesive-removal test and modified neurological severity score (mNSS) test compared to the MCAo + PBS group, at 14 days after MCAo [27]. However, we could not detect any functional recovery at 5 days in both groups.

Tissue extracts, from brain harvested for analysis, were prepared by homogenization with DEPC water (Sigma, St. Louis, MO). Brain samples were freshly obtained under fasting conditions and were immediately stored at −70°C until analysed. To exclude the possibility of variation in diet and other factors, we used rats that were fed the same diet and maintained in the same housing environment throughout the study period.

4.4. hBM-MSCs Culture. hBM-MSCs were provided from Pharmicell (Seoul, Korea) under culture in GMP (Good Manufacturing Practice) conditions as in our previous report [13, 28]. Briefly, cells were incubated at 37°C in 5% CO_2 in flasks for one day and nonadherent cells were removed by replacement of the medium. Once the cells reached about 80% confluence, they were harvested with 0.05% trypsin and 0.53 mmol/L EDTA (GIBCO, Grand Island, NY) for 5 minutes at 37°C, replated in a flask, cultured again for 3–5 days, and harvested. In order to achieve a sufficient dose, hBM-MSCs used in these experiments were collected from six passages [27].

4.5. Chemicals and Reagents. Putrescine, cadaverine, spermidine, spermine, N^1-acetylputrescine, N^1-acetylcadaverine, N^1-acetylspermidine, N^8-acetylspermidine, N^1-acetylspermine, 1,6-diaminohexane, ethylchloroformate (ECF), and pentafluoropropionyl anhydride (PFPA) were obtained from Sigma-Aldrich. Diethyl ether, ethyl acetate, and dichloromethane of pesticide grade were obtained from Kanto (Tokyo, Japan). Sodium chloride, obtained from Junsei (Tokyo, Japan), was washed successively with methanol, acetone, dichloromethane, and diethyl ether, followed by drying under a vacuum (100°C, 1 h). Sodium hydroxide was obtained from Duksan (Seoul, South Korea). All other chemicals were of analytical grade.

4.6. Sample Preparation of Brain Tissues. Sample preparation for assay of PAs in rat brain samples was performed according to our previous method [41] for the following brain samples: control, control + hBM-MSCs, MCAo, and MCAo + hBM-MSCs groups. Brain tissue in 5 mL distilled water was homogenized (3 min, 30,000 rpm) in an ice water bath using a model Pro 200 rotor/stator-type tissue homogenizer (Pro Scientific, Oxford, CT). An aliquot equivalent to 20 mg of brain tissue, including 1,6-diaminohexane (0.2 μg), as the internal standard (IS) of the homogenate, was vortex-mixed with 1 mL acetonitrile for 3 min. The mixture was centrifuged at 15,000 rpm and 15 min for protein precipitation. Briefly, ethoxycarbonyl (EOC) reaction was performed in aqueous phase by 10 min vortex with 20 μL ECF present in 1 mL of the dichloromethane phase. Then, the mixture was saturated with sodium chloride and sequentially extracted with diethyl ether (3 mL) and ethyl acetate (2 mL). The extracts were evaporated under a nitrogen gentle stream at 40°C, which were converted as PFP derivative (60°C for 30 min) with PFPA (20 μL) for analysis by gas chromatography-mass spectrometry (GC-MS) in the SIM mode (GC-SIM-MS) as described previously [41].

4.7. Sample Preparation of Cells. PA profiling analysis was performed, as previously described [41], in the following cell groups: control, oxygen-glucose deprivation (OGD), and OGD + hBM-MSCs groups. IS (0.1 μg) was added to each cell aliquot (4×10^5 cells) after freeze-thaw lysis, which were subjected to the aforementioned EOC-PFP reactions. The reaction mixtures were then analysed by GC-SIM-MS as described previously [41].

4.8. Exposure of SH-SY5Y Human Neuroblastoma Cells to Oxygen-Glucose Deprivation. SH-SY5Y cells (passage <~50) were cultured in an aerobic incubator with 5% CO_2 and humidified at 37°C. High glucose Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), was used for culturing. Oxygen-glucose deprivation was performed as described previously [42]. Briefly, the culture medium was replaced by glucose-free DMEM containing 10% FBS, and SH-SY5Y cells were adapted by incubation in an aerobic incubator for 4 h at 8×10^4 cells per cm^2. OGD cells were then transferred to an anaerobic chamber, containing a gas mixture of 95% N_2 and 5% CO_2, and humidified at 37°C. The medium was subsequently replaced by glucose-free DMEM that had been purged using N_2. After 24 h OGD incubation, OGD was terminated by removing the cultures from the chamber and replacing the medium with glucose-enriched DMEM. Other SH-SY5Y cells exposed to the OGD condition were also treated with 10^5 cells per cm^2 hBM-MSCs in the insert well using transwell for 72 h (OGD + hBM-MSCs). The OGD group without hBM-MSCs consisted of culturing aerobically for the same period of time. All samples were collected 72 h later.

4.9. GC-MS. As outlined in our previous report, GC-MS analysis in SIM mode for quantitative analysis of PAs in rat brain tissue was conducted with an Agilent 6890N gas chromatograph interfaced to an Agilent 5975 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (SE-54 bonded phase; 25 m × 0.20 mm I.D., 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Santa Clara, CA) [28].

4.10. Star Symbol Plotting. PA values were measured in cells. For each sample, PA values were normalized to the corresponding mean values in the normal group. Each normalized value was then plotted as a line radiating from a common

central point. The far ends of seven lines for cells were joined together to produce a star pattern for each group using the MS Excel program, as described in the previous report [43, 44].

5. Conclusion

Outcomes of our metabolomic experiments have provided new insight into our understanding of the complexity of biochemical and physiological events that occur in ischemic brain injury and the therapeutic effects of hBM-MSCs in treatment of stroke. More importantly, elucidation of PA metabolism after hBM-MSCs transplantation could guide the future development of effective measures or therapeutic strategies for stem cell therapy for protection against cerebral ischemia or reduction of ischemic injury.

Competing Interests

The authors declare no conflict of interests.

Authors' Contributions

Tae Hwan Shin and Geetika Phukan contributed equally to this work.

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Review Article

Functional Properties of Human Stem Cell-Derived Neurons in Health and Disease

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Stem cell-derived neurons from various source materials present unique model systems to examine the fundamental properties of central nervous system (CNS) development as well as the molecular underpinnings of disease phenotypes. In order to more accurately assess potential therapies for neurological disorders, multiple strategies have been employed in recent years to produce neuronal populations that accurately represent *in vivo* regional and transmitter phenotypes. These include new technologies such as direct conversion of somatic cell types into neurons and glia which may accelerate maturation and retain genetic hallmarks of aging. In addition, novel forms of genetic manipulations have brought human stem cells nearly on par with those of rodent with respect to gene targeting. For neurons of the CNS, the ultimate phenotypic characterization lies with their ability to recapitulate functional properties such as passive and active membrane characteristics, synaptic activity, and plasticity. These features critically depend on the coordinated expression and localization of hundreds of ion channels and receptors, as well as scaffolding and signaling molecules. In this review I will highlight the current state of knowledge regarding functional properties of human stem cell-derived neurons, with a primary focus on pluripotent stem cells. While significant advances have been made, critical hurdles must be overcome in order for this technology to support progression toward clinical applications.

1. Introduction

Technological innovations in cell culture models over the last decade have revolutionized the study of developmental and disease processes of the central nervous system. With the advent of induced pluripotent stem cells (iPSCs) and direct conversion techniques to create induced neurons (iNs), researchers now have the ability to examine cellular and molecular pathways in a completely human context with extraordinary genetic, pharmacological, and physiological control. For example, novel genetic engineering approaches such as zinc finger and TALE nucleases, as well as CRISPR/cas9, allow researchers to "correct" mutations in cell lines from diseased patients [1] or create targeted, disease-related mutations in "wild-type" cells [2]. Coupled with improved differentiation and specification of various neuronal and glial lineages, the analysis of *in vitro* phenotypes can be carefully tested alongside isogenic control lines in an unprecedented manner.

While much of the focus of iPSC research has been to dissect the complex molecular signaling pathways that underlie disease processes, neuroscientists must also consider the impact of genetic mutations and environmental exposure on the *functional* properties of neurons. As one of two electrically excitable cells in mammals, many disease-related phenotypes are thought to manifest as deficits in membrane excitability or synaptic communication between various neuronal populations. Many disorders such as autism, schizophrenia, and epilepsy are increasingly known as channelopathies or synaptopathies [3, 4], where proteins known to be involved with ion conductance or synaptic transmission are mutated or otherwise disrupted. The behavioral disturbances manifest in disorders of known etiology stemming from either monogenic (e.g., Rett syndrome and fragile X syndrome) or polygenic (e.g., Down syndrome) abnormalities have traditionally been thought to result from disruptions of a host of downstream effector functions, which until

recently were not linked to neuronal communication. However, these pathological signaling processes are beginning to be appreciated as causing disruptions in network function by altering spike generation, integration of synaptic potentials, and/or plasticity. While *in vitro* stem cell model systems have generally lagged behind *in vivo* studies, the complex interplay between the cell-autonomous effects of deficits in neuronal function and the impact on network processing are only beginning to be elucidated even in animal models. Thus, stem cell models are now poised not only to validate *in vivo* findings but also to develop novel hypotheses regarding human-specific pathways of development and disease. However, it is imperative that stem cell biologists recognize both the opportunities and the limitations of this system to understand disease pathophysiology.

In this review I will survey recent progress regarding the functional properties demonstrated for multiple types of stem cell-derived neurons including iPSCs and iNs. I will briefly introduce the concepts regarding passive and active properties of neurons to shed light on how physiology can be used to assess neuronal maturity and identity, with a focus on glutamatergic and GABAergic neurons. Furthermore, I will discuss the ability of functional analysis to dissect pathological processes related to human disease. For my purposes, studies using hESCs and/or iPSCs have demonstrated similar findings in terms of basic functional properties [17]. Therefore, with few exceptions where applicable, I will use the more general term human pluripotent stem cell-derived neurons (hPSNs) to refer to both populations.

2. Functional Maturation of hPSC-Derived Neurons

2.1. Passive and Active Membrane Properties. The functional properties of neurons and their progenitors are driven by ion-conducting channels activated by a variety of stimuli including voltage fluctuations, secreted ligands, and mechanical forces (e.g., stretch). It is now well-established that, using differentiation techniques developed by either Zhang and colleagues [18] or Chambers and colleagues [19], a proportion of postmitotic cells display the functional hallmarks of (relatively) immature neurons compared with *in vivo* reports. The vast majority of reports using these methods suggest that, without directing specification toward particular lineages, hPSNs differentiate to forebrain progenitors which will then produce a mixed population of cortical-like glutamatergic and GABAergic neurons [20, 21], upon which we will focus our discussion. In addition, relatively pure populations of excitatory glutamatergic neurons and GABAergic interneurons can be specified using exogenous morphogens (see below) but generally express similar functional properties to "default" differentiated hPSNs.

Passive membrane properties are primarily determined by three features: (1) conductance of nongated or "leak" ion channels, (2) membrane capacitance (C_m), and (3) conductance of the cytoplasmic milieu. At present we will ignore cytoplasmic milieu as this is not measured using standard patch clamp techniques. Commonly, ion channel conductance (g) is measured indirectly as the cellular input resistance (R_{in}), which is simply the inverse of the conductance ($g = 1/R_{\text{in}}$). Passive membrane properties are defined as those that remain *constant* during signaling processes such as action potential generation or synaptic activity. However, this is only true for a static system, most often found in "mature" neurons; developing cells display significant changes in C_m as neurons add new plasma membrane to elongating neuronal arbors. This has been demonstrated consistently for hPSNs, where C_m values significantly increase with time in culture or with addition of astrocytes (see below). R_{in} can generally be thought of as the number of channels per unit area of membrane and typically decreases as neurons mature, indicating the progressive addition of channels to the plasma membrane. For hPSNs, while C_m and R_{in} show robust changes over culture duration, values typically resemble neurons of late fetal stages, but not adult neurons *in vivo* and from primary cultures. For instance, with few exceptions [22], C_m values are twofold to fivefold lower, while R_{in} measurements for hPSNs are fivefold to tenfold higher than primary rodent neurons [21, 23]. This may simply reflect the relatively early time points used in most studies, as hSPNs recorded at 30 weeks *in vitro* demonstrate mean C_m values above 50 pF and R_{in} values close to mature rodent counterparts [24].

The resting membrane potential (RMP) of a cell is another proxy for determining neuronal maturity. Multiple reports have demonstrated that RMP decreases for hPSNs over prolonged periods in culture and can reach relatively mature levels after several months [7, 21, 25]. Of note regarding RMP reporting, there is wide discrepancy in the literature regarding the use of liquid junction potential (LJP) compensation to adjust RMPs based on differential ionic concentrations in the intracellular and extracellular recording solutions [26]. This can lead to wide disparities in reported RMPs, as LJP compensation can shift RMP values by 10 to 15 mV (nearly always in the negative direction) depending on solutions used. We recommend the use of LJP compensation and/or parallel recordings from primary neurons [23] to validate that RMPs recorded are accurate. RMP is largely determined by the conductance of ions through leak or nongated channels, in particular potassium (K^+) channels in neurons. The identity of K^+ leak channel in rodent neurons remained unknown until 1995 despite the cloning and/or characterization of most voltage- and ligand-gated channels. However, it is now known that a large family of two-pore forming KCNK channels is largely responsible for K^+ conductance that drives RMP [27]. Because RMP of developing hPSNs decreases with time in culture, this conductance likely increases relative to C_m. Although a functional demonstration has not been reported, gene expression studies support this notion as *KCNK3* and *KCNK10* demonstrate increasing transcript abundance over time [28].

The interplay between leak and voltage-gated potassium and sodium channels determines the intrinsic active membrane properties of neurons, including their ability to generate action potentials (APs). Nearly all studies that have quantified AP generation in hPSNs demonstrate progression from relatively immature spiking phenotypes at early time points to repetitive spike firing with more mature AP characteristics. In addition to increased number of spikes

per train, AP maturation includes larger amplitude, shorter AP half width (faster spikes), and lower spiking threshold potentials [29]. However, even for the most mature hPSNs, individual APs still show prolonged half widths, moderate amplitudes, and fewer spikes/train compared with *in vivo* reports. Interestingly, the development of voltage-gated currents underlying spike generation is very similar to *in vivo* studies. For instance, voltage-gated K^+currents are prevalent in the early developing cortex and are consistently found in progenitor cells of the ventricular and subventricular zones [30, 31]. As progenitors exit cell cycle and differentiate, only small increases are observed for total K^+ currents, whereas the fraction of total current contributed by sustained (I_K) and transient (I_A) currents changes [30, 32]. I_A current is largely responsible for rapid repolarization during spike firing, allowing for repetitive AP generation. Similar to *in vivo* data, hPSNs display progressive increases in I_A current with time in culture, and this parallels their ability to generate repetitive AP trains [21]. Unlike v-gated K^+currents, progenitor cells typically lack v-gated sodium (Na^+) currents (I_{Na}, [30, 31]). However, even early postmitotic neurons demonstrate relatively robust I_{Na} currents [8], which show modest increases with prolonged culture periods [21, 25, 33]. Together, higher R_{in} and RMPs, as well as lower C_m, render hPSNs relatively more excitable than their mature counterparts. Thus, despite significantly smaller mean I_{Na} currents compared to adult neurons *in vivo*, hPSNs are able to fire APs in response to smaller current injections, and a substantial portion are intrinsically active. In addition, a small minority of hPSNs display properties similar to neurons *in vivo*, such as I_{Na} currents larger than 10 nA, LJP-adjusted RMPs near −70 mV, and C_m values greater than 70 pF ([34], Weick, unpublished observations). However, the variability across individual hPSNs, coverslips, differentiation methods, and laboratories leads to mean values resembling immature cells typical of late embryonic stages in rodents.

2.2. Glutamatergic Transmission. Rapid excitatory neurotransmission in the brain is primarily mediated by the glutamatergic *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. AMPARs mediate relatively fast depolarization primarily via Na^+ influx and are composed of tetrameric assemblies of GluA1-4 subunits [35]. The GluA2 subunit is unique in that it undergoes an ADARB1-dependent posttranscriptional modification that alters the mRNA sequence encoding amino acid 607 (glutamine (Q)) to a codon encoding arginine (R) in the M2 pore forming region of the channel. Q/R editing confers several important properties to AMPARs when edited GluA2 subunits are present, including calcium (Ca^{2+}) impermeability, insensitivity to block by polyamines, and reduced single channel conductance [36]. Within the hippocampus and cerebral neocortex, the vast majority of AMPA receptors contain edited GluA2, which predominantly forms heteromers with GluA1 [37, 38]. However, some reports suggest that, during early development, unedited GluA2 subunits predominate, with edited subunits being exchanged during maturation [39]. To estimate incorporation of edited GluA2 in hPSNs, Livesey et al. (2014) analyzed AMPA-evoked currents to estimate mean single channel conductance at two time points. They found significantly reduced AMPAR conductance after 5 weeks following plating compared to 2-week-old neurons. In addition, compared with five-week-old cells, neurons plated for only 2 weeks showed significantly greater sensitivity to the spermine. Together, these data suggest a developmental shift consistent with incorporation of edited GluA2 in older hPSNs [40]. Interestingly, GluA2 mRNA expression greatly exceeds that of other GluA subunits, while levels of *ADARB1* increase during hPSN differentiation [28], suggesting its expression is rate limiting for GluA2 editing consistent with previous *in vivo* studies [39, 41].

In contrast to AMPARs, NMDARs are not required for synaptic transmission in mature neurons of most brain regions but appear to play significant role in triggering the changes that underlie plasticity. This property is due largely to their ability to conduct Ca^{2+}, which acts as a second messenger through activation of various downstream kinase and phosphatase cascades [42]. Similar to AMPARs, NMDARs are also thought to be comprised of tetrameric assemblies of subunits but show a strong requirement for incorporation of the NR1 (GluN1) subunit, with variable incorporation of the NR2 subunits (GluN2A-D) and/or NR3 (GluN3A-B) subunits [43]. At various excitatory synapses, including from thalamic and cortical neurons, developmental studies have shown a switch from primarily NR1/NR2B-containing to NR1/NR2A-containing receptors [44, 45]. The various subunit combinations confer critical properties to NMDARs, where NR2B-containing receptors demonstrate significantly prolonged currents and greater sensitivity to various blockers [43]. A similar developmental switch occurs for NR3 subunits, whereby NR3A predominates during embryonic and early fetal periods, and NR3B expression increases throughout adulthood [46, 47]. Using dual-SMAD inhibition in the presence of the sonic hedgehog inhibitor, Dolmetsch and colleagues demonstrated robust NMDAR currents in iPSNs [12]. Similar results were found by Gupta et al. (2013) for hESNs, which show glutamate-induced toxicity that is blocked by NMDAR antagonist MK801 [48]. Lastly, a recent study from Livesey and colleagues reported that NMDA-dependent synaptic plasticity could be induced in hPSN cultures using NMDAR activation. Fifty minutes following removal of extracellular Mg^{2+} and treatment of cultures with glycine, hPSNs were found to increase synchronicity and amplitude of spontaneous bursting, which could be blocked by AP5 [49]. These are the first data to support the idea that *in vitro*-generated hPSNs can undergo long-term changes in synaptic efficacy.

2.3. GABAergic Transmission. Inhibitory neurons of the CNS that express the neurotransmitter GABA come in two major flavors: (1) projection neurons and (2) interneurons (INs, not to be confused with induced neurons (iNs)). GABAergic projection neurons include the medium spiny neurons (MSNs) of the basal ganglia and Purkinje neurons of the cerebellum, which make long-range connections between distant brain regions. GABAergic INs occupy nearly all brain regions to varying degrees and make contact with excitatory neurons and other inhibitory INs locally within those areas.

While INs represent only about 20% of the mammalian cortex, they are primarily responsible for maintaining excitatory-inhibitory balance in local circuits. For instance, disrupted IN function has been implicated in multiple neurological disorders including Alzheimer's, autism, epilepsy, and schizophrenia [50–53].

Early functional studies of hPSNs revealed the presence of spontaneous inhibitory synaptic activity in default differentiated cultures [21]. Gene expression [28] and immunocytochemical analyses [7] confirmed the presence of a host of genes involved with IN specification, but the precise nature of these GABAergic neurons remains to be determined. Reports using a NKX2.1-GFP reporter line have demonstrated highly enriched populations of GABAergic INs using combinations of WNT antagonism paired with treatment of progenitors with the ventralizing morphogen sonic hedgehog (SHH) [24, 54]. In contrast to default patterned cells that lack Nkx2.1 and a majority of mature IN markers [55], direct differentiation produced multiple markers of the medial ganglionic eminence (MGE), the primary origin of cortical INs [56]. In addition these cultures showed robust diversity of IN phenotypes from Nkx2.1$^+$ progenitors. Postmitotic neurons expressed a range of general IN markers such as ASCL1 and DLX2 and, at later stages of development, expressed subtype-specific markers such as calretinin (CALB2), parvalbumin (PV), and somatostatin (SST). Furthermore, significant expression of functional inhibitory markers such as *GAD1* (*GAD67*), *SLC32A1* (*VGAT*), and *SLC6A1* was observed at relatively later time points. In addition, expression analysis [28] and pharmacological dissection of $GABA_A$ currents themselves [57] suggest that hPSNs up to 7 weeks of age primarily express $GABA_A$Rs comprised of the α2/3-β3-γ2 subunits, which is the predominant composition in the embryonic cortex [58]. This lies in contrast to more mature neurons that primarily express α1-subunit-containing $GABA_A$Rs [59].

Similar to excitatory neurons, default differentiated GABAergic neurons and directed INs display progressive increases in spiking frequency and amplitude, reduced AP half width, and increased synaptic activity that is sensitive to $GABA_A$R antagonists picrotoxin and bicuculline. Functional INs also display relatively immature excitable properties compared to their *in vivo* counterparts and, in some reports, appear even more delayed than their excitatory counterparts [24]. *In vivo*, INs that occupy the cortex and hippocampus display the broadest range of spiking phenotypes of any neuronal population, including regular spiking (typically 10–20 Hz), fast spiking (>30 Hz), irregular spiking, delayed spiking, bursting, and stuttering [56, 60]. However, the vast majority of hPSN-derived INs appear to demonstrate regular spiking phenotypes with a small minority displaying delayed spiking properties [61]. This may be a result of deficient network activity *in vitro* where INs lack appropriate innervation from sensory fibers. One prevailing hypothesis for IN development is that while subtype specification of INs occurs transcriptionally during differentiation of MGE/CGE progenitors, maturation of functional IN properties depends on innervation from presynaptic neurons and can be highly specific to IN subtype [56, 62]. This so-called "learning on the job" may be required for hPSN-derived INs of various subtypes to achieve fully functional status.

2.4. Role of Glial Cells. In primary neuronal cultures as well as *in vivo*, previous studies have demonstrated that astrocytes play a significant role in promoting neuronal maturation, specifically through effects on synaptogenesis [63]. Reports using stem cell-derived neurons corroborate these findings and suggest additional roles for glia in promoting neuronal maturity. First, Johnson et al. (2007) showed that significant numbers of astrocytes differentiate from default patterned forebrain progenitors around 6-7 weeks in culture. This was coincident with the appearance of excitatory and inhibitory spontaneous postsynaptic currents (sEPSCs and sIPSCs) in hPSNs. Further, hPSNs grown on mouse cortical astrocytes developed sEPSCs/sIPSCs at significantly earlier time points compared to cultures without astrocyte addition [21]. Chen and colleagues extended these studies to show that mouse cortical astrocytes enhanced survival, arborization of neurites, AP firing, and sEPSC/sIPSC frequency and amplitude. One of the more dramatic findings from this study was the glial-induced increase in capacitance (pF); hPSNs grown for 60 days without astrocytes displayed typical C_m around 27 pF, whereas those in coculture achieved values around 120 pF, values more typical of primary rodent cortical and hippocampal neurons [34]. While this is likely due in part to increased dendritic complexity, it is unclear whether these results are unique to particular cell types, as other reports have shown more modest increases in hPSN capacitance in the presence of astrocytes [23].

2.5. In Vitro Network Properties. The vast majority of reports to date have focused on hPSN functions with patch clamp on a single cell level [21, 25, 33]. However, the ability of these cells to form spontaneously active networks is of great interest for conducting large-scale *in vitro* drug screening, toxicology studies, and understanding disease pathology on whole network properties. Interestingly, it was reported that networks derived from default differentiated cells rarely generate spontaneous synchronized bursts but are capable of adopting network activity when cocultured with dissociated mouse cortical neurons. It has been suggested that the lack of bursting in hPSNs may be due to the presence of a significant proportion of inhibitory interneurons [23]. This idea has been supported by reports using various secreted factors to drive glutamatergic differentiation, where more pure populations of excitatory hPSNs are capable of network bursting [49, 64]. However, it may be the case that a relatively sparse population of excitatory neurons is not capable of generating bursts or that patch clamping of individual cells is insufficient to detect relatively sparse or infrequent network activation, whereas Ca^{2+} imaging of larger numbers of cells is more sensitive ([49] but see [65]).

To identify network activation in a high throughput manner, multiple laboratories have employed multielectrode array (MEA) recording platforms. Initial studies of murine ESC-derived neurons showed that the cells were capable of forming spontaneously active networks [66, 67]. Similar to studies using whole-cell patch clamping, activity was observed to

progressively develop from single spikes into more complex trains, followed by bursting [66, 67]. Validation of human ES cell-derived neuronal network formation via MEA recordings followed shortly thereafter [68]. Similar to murine systems, early forms of activity take the form of single spikes detected on various nodes that are randomly distributed; these spikes reflect axonal and/or dendritic signaling in the developing network. As the network matures, train-like spiking can give way to synchronous bursting, which is considered as mature signaling activity of the network [68, 69]. Network activity of hPSNs and mESC-derived neurons appears to be driven by excitatory and inhibitory synaptic activity as these cultures respond to AMPA/kainate, NMDA, and GABAA receptor blockers [67, 68]. Interestingly, bursting was observed on a minority of recording electrodes within the MEA platform, and those nodes that recorded bursting were clustered but also widely distributed. Thus, it may be the case that local networks within a culture are able to form bursting networks while others remain nonfunctional or simply display unique network properties. In any case, MEA recording provides a powerful tool to dissect the effects of cell type specificity, genetics, environmental exposure, and differentiation methodologies on the functional development of network behavior.

2.6. Functional Properties of Induced Neurons (iNs). Despite the substantial benefits of iPSC systems, they do have significant limitations including inefficiency (typically fewer than 1% of cells are reprogrammed) and time intensity (reprogramming and differentiation typically take 3-4 months). In addition, pluripotency is associated with genetic instability and tumorigenesis [70]. To overcome these issues, direct conversion of somatic cells has been used to generate functional neurons from wild-type and diseased tissue. First developed *in vitro* using fibroblasts [71], multiple reports have also demonstrated *in vivo* conversion of astroglial cells [72, 73], which may be useful as an alternative to cell replacement therapies for regenerative purposes. The functional properties of multiple reports of human iNs (hiNs) have been summarized nicely by Chinchalongporn and colleagues [29], who suggest that most *in vitro* studies using a wide variety of combinatorial reprogramming factors report maturation levels similar to hPSNs, especially for adult somatic cell reprogramming, based on a host of passive and active properties. For instance, despite the fact that some hiNs can fire action potentials as early as 8 days after conversion, most hiNs display RMPs at relatively depolarized potentials (greater than −40 mV), low C_m (<40 pF), and high R_{in} values (1-2 MΩ). In addition, most reports use cocultures of iNs with primary neurons or astrocytes to induce synapse formation; a convincing demonstration of synapse formation using conversion of adult cells without cocultures remains elusive. In contrast, iNs converted from embryonic stem cells using single transcription factors show functional properties more similar to *in vivo* counterparts with rapid development of spiking and synaptic activity. Multiple reports have demonstrated the utility of iNs derived from stem cells to model diseases such as those associated with neuroligin-3 [74] and neurexin-1 mutations (see below, [2]) that may underlie various neurological disorders such as autism.

3. Functional Deficits in Neurological Disease

Multiple neurological disorders are thought to arise due to alterations in functional properties including developmental disorders such as autism spectrum disorders (ASD), Down syndrome, Dravet syndrome [8–10], Rett syndrome, schizophrenia (SCZ), and neurodegenerative disorders such as Alzheimer's (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and spinal muscular atrophy (SMA). Table 1 summarizes many recent examinations of functional phenotypes found in diseased hPSNs. A unifying theme has begun to develop for many of these disorders, focused on the concept of excitation-inhibition balance as an endpoint to circuit-level dysfunction. While the etiology of particular disorders may lie with individual gene deficits (e.g., mutation of the MECP2 gene in Rett syndrome), the ultimate expression of dysfunction lies at the level of neuronal excitability and synaptic integration. Recent studies of human stem cell-derived neurons have identified multiple functional deficits that validate other preclinical models and, in some cases, appear highly specific.

One of the earliest examinations using hPSNs to model functional deficits was performed by Paşca et al. (2011) to study the effect of a missense mutation of the voltage-gated Ca^{2+} channel CACNA1C ($Ca_v1.2$), which causes Timothy syndrome [15]. While the most severe phenotype associated with Timothy syndrome is cardiac arrhythmia, patients also suffer from developmental delay [75]. Neurons carrying $Ca_v1.2$ mutations displayed a significantly prolonged AP duration as well as greater elevations in sustained intracellular $[Ca^{2+}]_i$. As Ca^{2+} acts as a second messenger to trigger long-term changes in cellular function, Timothy syndrome neurons displayed significant alterations in depolarization-induced gene expression compared with controls. These changes were correlated with differences in neuronal differentiation both *in vitro* and compared to transgenic mice carrying $Ca_v1.2$ mutation.

Using MEA recordings, Woolf and colleagues examined the spontaneous firing properties of motor neurons derived from patients with ALS carrying $SOD1^{A4V}$ mutation compared with unrelated wild-type cells. ALS hPSNs showed significantly greater spiking with no changes observed for passive membrane properties, which could be corrected via genetic correction of $SOD1^{A4V}$ mutation. Interestingly, delayed rectifier potassium currents driven by the KCNQ family of Kv7 channels were markedly reduced in ALS hPSNs, and administration of the Kv7 agonist retigabine reduced hyperexcitability and caused marked hyperpolarization (~6 mV) with EC50 of 1.5 μM (Table 1). Gene expression analysis suggests that the KCNQ2 channel was likely responsible for these effects, consistent with expression in cortex [5, 28]. However, previous reports have not identified the KCNQ family as linked to ALS, which may suggest a human-specific effect using ALS hPSNs. Interestingly, hyperexcitability may be a common mechanism underlying

Table 1: Dysfunction and treatment of diseased human stem cell-derived neurons.

Disease	Cell type(s)	Observed phenotypes	Refs	Treatment
Amyotrophic lateral sclerosis (ALS)	iPSC-derived motor neurons	Motor neurons derived from ALS iPSCs displayed hyperexcitability	[5]	Kv7 channel-activator retigabine reversed MN hyperexcitability
Bipolar disorder	Forebrain hPSNs	Increased AP frequency and amplitude in lithium-responsive and -nonresponsive hPSNs selectively responded to treatments (column 5)	[6]	Li^{2+} reduced hyperexcitability in hPSNs from Li^{2+}-responsive patients Lamotrigine reduced hyperexcitability in Li^{2+}-nonresponsive hPSNs
Down syndrome	Forebrain hPSNs	Decreased frequency (not amplitude) of spontaneous excitatory and inhibitory synaptic events	[7]	None reported
Dravet syndrome	Forebrain hPSNs	(i) Spike generation impaired in GABAergic neurons	[8]	Phenytoin reduced hyperexcitability
		(ii) Increased sodium currents	[9]	
		(iii) Hyperexcitability/spontaneous bursting resembling epileptiform activity	[10]	
Huntington's disease	Forebrain and striatal hPSNs	CAG repeat length-dependent reductions in spiking associated with increased cell death	[11]	None reported
Phelan-McDermid syndrome (22q13 deletion)	Forebrain hPSNs	Selective reduction in amplitude and frequency of spontaneous excitatory postsynaptic currents (excitation-inhibition ratio altered)	[12]	Genetic expression of Shank3 or IGF1 treatment restored EPSCs
Psychiatric disease (ASD/SCZ) (NRXN1 mutants)	Forebrain hPSNs and iNs	Impaired neurotransmitter release; reduced sEPSC frequency upregulation of presynaptic CASK protein	[2]	None reported
Rett syndrome	Glutamatergic hPSNs	Decreased activity-dependent calcium oscillations Reduced frequency and amplitude of spontaneous synaptic currents	[13]	None reported
Spinal muscular atrophy (SMA)	iPSC-derived motor neurons	Hyperexcitability and impaired neurotransmission Greater R_{in} and lower voltage threshold for spike induction	[14]	Genetic correction reversed phenotypes
Timothy syndrome	Forebrain hPSNs	Increased action potential width Greater elevations of intracellular calcium	[15]	None reported
Williams-Beuren syndrome	Forebrain hPSNs	Reduced AP amplitude and prolonged decay; no effect on other passive/active conductance nor mEPSCs	[16]	None reported

motor neuron disorders. Liu and colleagues differentiated motor neurons derived from hPSNs that carry mutations/deletions of the survival of motor neuron (SMN) genes, which lead to spinal muscular atrophy (SMA). SMA neurons displayed lower threshold of AP generation, larger spike amplitudes, and greater frequencies. In addition, SMA motor neurons showed enhanced I_{Na} currents with faster recovery rates, all of which were restored by expression of wild-type SMN [14]. In contrast to increased spike firing of mutant hPSNs, Kinnear and colleagues found that AP amplitude was reduced while decay was prolonged in hPSNs from Williams-Beuren syndrome iPSCs, caused by a deletion of ~25 genes on chromosome 7 [16]. Similarly, hPSNs derived from HD patients [11, 76] that display extended CAG repeats (e.g., 180) in the huntingtin gene showed reductions in spike generation that correlated with increased cell death [11]. Thus, functional phenotypes that span the spectrum from hyper- to hypoexcitability can be modeled using hPSNs to gain insight into pathological features of disease.

In addition to spiking phenotypes, multiple reports have found deficits at the synaptic level that may underlie various neurological disorders. For instance, in neurons derived from iPSCs from patients with either Rett syndrome or Phelan-McDermid syndrome (PMDS), excitatory neurotransmission was impaired as indicated by reduced amplitude and frequency of sEPSCs [12, 13]. In PMDS neurons excitatory

transmission was selectively impaired, leading to a loss of E/I balance. However, E/I balance could be restored via genetic overexpression of Shank3 or treatment of cultures with IGF1, which selectively increased sEPSC amplitude and frequency [12]. In contrast, the frequency of both sEPSCs and sIPSCs was equally diminished in iPSC-derived neurons from patients with trisomy 21 (the cause of Down syndrome) [7], leading to quieter network activity overall. Importantly, physiological data was correlated with reduced immunocytochemical labeling of presynaptic synapsin-1 protein, suggesting an impairment of synaptic development regardless of transmitter phenotype. Similarly, Sudhof and colleagues used both hPSNs and induced neurons (iNs) to model psychiatric diseases (e.g., ASD and SCZ) by creating heterozygous conditional neurexin 1 (NRXN1) mutations in human embryonic stem cells (hESCs). They found that heterozygous loss-of-function NRXN1 mutations had no effect on neuronal differentiation and synaptogenesis, because labeling of Syn1 was comparable to control neurons. However, NRXN1 mutant neurons severely impaired neurotransmitter release in a stimulus-dependent manner. Interestingly, this phenotype was specific to human neurons as mouse Nrxn1a mutations exhibited no phenotype [2].

More recently, Mertens and colleagues examined the phenotypes of hPSNs differentiated from cells taken from patients with type I bipolar disorder (BD, [6]). In an elegant design this study derived cells from BD patients that showed clinical responsiveness to Li^{2+} (LR) and those that were nonresponsive (NR). Interestingly, both populations of hPSNs showed hyperexcitable properties, with increased spontaneous and evoked AP frequency and amplitude, lower threshold of activation, and increased I_{Na} compared to control hPSNs. Interestingly, treatment of cultures with 1 mM LiCl eliminated these differences selectively in hPSNs derived from LR patients, but not from NR patients. However, cells from NR patients did show responsiveness to the antiepileptic drug lamotrigine. The authors went on to characterize the gene expression changes induced by Li^{2+} treatment and found several potential pathways altered, including genes involved with energy homeostasis and mitochondrial function, PKA/PKC signaling, and K^+ channels.

4. Conclusions and Perspectives

Since the creation of iPSCs in 2007 and iNs in 2010, they have been used to examine disease phenotypes in hundreds of publications. Thus, in a short time these platforms have had a significant impact on our understanding of disease pathology and treatment and will likely change the direction of translational research going forward. With respect to functional phenotypes, it is remarkable that fully *in vitro*-generated cells can recapitulate many aspects of disease with high degrees of specificity (Table 1). hPSNs and iNs have been shown to express a wide array of voltage-dependent and independent ion channels, the appropriate ligand-gated receptors for various neurotransmitters, and the ability to form spontaneous neural networks as well as integrate with established networks from animals separated by millions of years of evolution. In addition, reports are now emerging to suggest that signaling mechanisms in hPSNs exist to alter synaptic efficacy, a critical factor in determining fully functional neural networks. Thus, the number of conserved developmental processes that exist *in vitro* supports the furthered use of hPSNs and iNs to uncover novel and potentially human-specific molecular pathways governing functional maturation, dysfunction, and degeneration.

However, with any new tool we must caution against overinterpretation of the significance of any individual finding, particularly in light of the immature nature of the neurons derived and the variability of timing and cell type developed. As others and I have indicated [77], hPSNs and iNs achieve functional phenotypes that resemble fetal and early postnatal rodent neurons. High throughput transcriptomic studies largely agree with these findings, showing that some of the more mature hPSNs reported to date display expression profiles similar to midgestational human fetal brain tissue [78]. In addition, in many differentiation paradigms only a small percentage of neurons display synaptic markers (2–5%, [12]), and most cultures still contain a large population of progenitor cells, which do not exist in most brain regions of adults. Furthermore, iN cells typically require the use of cocultured astrocytes or primary neurons to form functional synapses [2], and direct conversion of adult cells produces neurons that are functionally less mature than those produced from neonatal cells. Interestingly, iNs from older cells appear to retain transcriptional programmes of older cells, while conversion into iPSCs typically eliminates age-related epigenetic signatures [79]. Thus, it will be critically important to improve neuronal maturation of adult-derived iNs before employing them in the study of age-related disorders. In addition, while coculturing healthy and "diseased" cells together can assist with understanding the cell-autonomous versus global network deficits, the presence of healthy cells may mask functional deficits due to paracrine and contact-mediated alterations in synaptic development. Table 2 compares some of the main features of hPSNs and iNs to consider for experimental design.

With respect to variability in hPSN, efforts are currently underway to generate single cell transcriptomic and morphological signatures to correlate with functional properties of hPSNs in an effort to help identify subclasses of neurons. Through combined use of directed differentiation, cell sorting, and genetically encoded reporter lines, derivation of pure populations of transmitter- and functional phenotype-specific neurons is an achievable goal for some laboratories. These techniques will be particularly useful for cell-based therapies. Together with continued improvements in reducing tumorgenicity and aberrant differentiation through the use of insertion-free approaches and screening, iPSCs appear poised to revolutionize replacement therapies via functional integration with appropriate neural circuits. And, despite the cautionary notes, it is exciting to see that many stem cell researchers recognize the importance of functional assays as a complement to biochemical studies. With the improvements noted here and elsewhere [29, 80], developmental and disease modeling with human stem cells have the potential to break new ground for patient-specific therapies as well as uncover

Table 2

Cell type	hESCs (primed)	iPSCs (primed)	iNs
Efficiency	>90%	Variable (up to 90%)	Low (2–11%)
Time to functional maturity	5 weeks +	5 weeks +	2 weeks +
Epigenome status	Embryonic (open)	Some adult modifications retained	Adult modifications maintained
Cell types produced	Neurons (many subtypes), astrocytes, oligodendrocytes	Neurons (many subtypes), astrocytes, oligodendrocytes	Primarily glutamatergic neurons
Purity of phenotypes	Heterogeneous (<80% pure)	Heterogeneous (<80% pure)	Relatively pure (>80%)
Effect of astrocytes	Accelerates maturation	Accelerates maturation	Required for functional maturation
Genetic intervention	N/A	Required	Required
Developmental studies	Appropriate	Appropriate	Less appropriate
Culture duration	Months	Months	Weeks

unifying mechanistic insights into seemingly disparate disease pathologies.

Competing Interests

The author declares having no competing interests.

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Review Article

Human Embryonic Stem Cells: A Model for the Study of Neural Development and Neurological Diseases

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Although the mechanism of neurogenesis has been well documented in other organisms, there might be fundamental differences between human and those species referring to species-specific context. Based on principles learned from other systems, it is found that the signaling pathways required for neural induction and specification of human embryonic stem cells (hESCs) recapitulated those in the early embryo development *in vivo* at certain degree. This underscores the usefulness of hESCs in understanding early human neural development and reinforces the need to integrate the principles of developmental biology and hESC biology for an efficient neural differentiation.

1. Introduction

Development of the vertebrate central nervous system (CNS) is one of the earliest events in embryonic germ layer induction, and it has long been thought of as a step following the formation of the embryonic ectoderm [1]. This development involves multiple steps, beginning with the induction of neuroepithelium from the embryonic ectoderm and completing with the patterning of different parts of the brain. The CNS is a complex tissue, in terms of both the number of cells and the variety of cell types. In addition, billions of neurons have to interact in a very precise manner in order to form functional neuronal networks. The CNS is formed over time during embryogenesis and is rapidly converted from simple neural plate to a brain and spinal cord. To form a many different types of neurons and glial cells in the adult CNS, embryonic cells have to proliferate and differentiate in a strictly controlled manner, and during the last few years rapid progress has been made in understanding the molecular mechanisms underlying the initiation, proliferation, and differentiation of the CNS [2]. Mice, chicken, and zebrafish have long been considered model organisms for the study of vertebrate development. Studies of these organisms have provided details into the molecular mechanisms underlying embryonic development and are beginning to suggest potential pathophysiological mechanisms of some important development/congenital abnormalities in humans. However, in the ultimate quest to understand the mechanisms of human development with the goal of preventing and treating developmental defects in humans, these studies fall short. Understanding molecular interactions underlying human development is limited by the availability of human embryos and inadequate amount of stage-specific and cell type-specific materials. These problems may now be solved by the uses of human embryonic stem cells.

2. The Properties of Human Embryonic Stem Cells

Human pluripotent stem cells (hPSCs), here including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs), are capable of expanding indefinitely and differentiating into all human germ layers

both *in vitro* and *in vivo* [3, 4]. During embryonic development, pluripotent stem cells are present only transiently and quickly differentiate into various somatic cells through developmental process [5]. However, it is possible to isolate *ex vivo* pluripotent mouse and human embryonic stem cells from the inner cell mass of blastocyst embryos and maintain them in laboratory. Human embryonic stem cells (hESCs) have the ability to renew and maintain their developmental potential to contribute to derivatives of all three germ layers, even after prolonged undifferentiated proliferation and/or clonal derivation [6]. In addition, hESCs can give rise to extraembryonic lineage, including trophectoderm and primitive ectodermal-like cells [4, 7]. Interestingly, hESCs are capable of expressing high level of telomerase, alkaline phosphatase, and key transcription factors that were also identified as being important in the maintenance of the inner cell mass pluripotency [8, 9]. These factors include the POU-family transcription factor OCT4, a homeodomain DNA-binding protein NANOG, and the SOX-family transcription factor SOX2. The embryonic markers defined by the antibodies SSEA-3 and SSEA4 are expressed by hESCs as well as the cell surface proteoglycan recognized by several monoclonal antibodies, including TRA-1-60 and TRA-1-81 [10]. The success derivation of hESCs provides a unique opportunity to study early human development and is believed to hold a great promise for biopharma and regenerative medicine [11, 12]. It is noted that the differentiation of hESCs in culture follows the hierarchical set of signals that regulate embryonic development in the generation of the germ layers and specific cell types at certain degree [13, 14]. Moreover, due to the difficulty of access to early human embryos and inadequate amount of stage-specific and cell type-specific materials, hESCs seem to provide a valid model to understand complex signaling interactions occurring in human embryos. In particular, the ability of hESCs to differentiate into defined neural lineages, neurons, astrocytes, and oligodendrocytes, is fundamental to study the sequence of events that take place during early neurodevelopment [14, 15]. Altogether, hESCs are a suitable and valid system to address the significant roles of the signaling pathways involved in neural lineage commitment and, ultimately, to model pathology of neurological disorders.

3. The Promising Applications of Human Embryonic Stem Cells

Although the success in establishing hESCs raised numerous ethical concerns, involving the development, usage, and destruction of human embryos, hESCs provide an alternative useful cell source for several potential applications in both basic science and medical treatment. To direct hESC differentiation *in vitro* along chosen pathways would allow for the investigation of early human development events, including regulatory signals for cell commitment and morphogenesis. Additionally, the cells could also be used for the screening of candidate drugs and carcinogenic or toxic compounds that cannot be analyzed in human embryos due to ethical constrains. However, investigations into the potential utility of hESCs in treating human diseases are at an infant stage because there are several issues needed to be taken into account, that is, efficiency, safety, and functionality [12].

The most urgent problem today in regenerative medicine is the lack of suitable donor organs and tissues. The pluripotent developmental potential of hESCs and the success of transplanting their differentiated derivatives into animal disease models reinforce the promising application of this cell type. This evidence has proofed the principle of using hESC-derived specific cells as a regenerative cell source for transplantation therapies of human diseases [16, 17]. One of the key issues causing hESCs technology to be useful for cell and tissue therapy in humans is the histocompatibility between graft and host. Recent data support the concept that hESCs and their differentiated derivatives possess immune privileged properties [18], suggesting that cells derived from hESCs may provide a potential tool for induction of immunotolerance [19]. On the basis of maternofetal immunotolerance observed during pregnancy along with the aforementioned immune privileged properties that ESCs share, the question of whether hESCs and their progeny can be considered potential vectors for tolerance induction in allogeneic recipients needs to become an area of active investigations [20, 21].

In another scenario for which the term "personalized pluripotent stem cells" has been coined, people could use their own somatic cells to be reprogrammed back to the pluripotent stem cell state. The feasibility of reprogramming was first demonstrated by somatic cell nuclear transfer (SCNT) or cloning. Somatic cells of patients are fused with enucleated oocytes; thereafter, hESCs could be established in culture and be induced to *in vitro* differentiation to provide patient-specific cells and tissues [22]. However, the reprogramming of somatic nucleus in an oocyte is still inefficient process. In addition, to access a source of human oocytes is not only a rare opportunity, but also an ethical concern worldwide [23]. As an alternative to reprogramming by SCNT, adult human fibroblasts can be directly reprogrammed into a state that is similar to hESCs by expression of only four factors, OCT4, KLF4, SOX2, and c-Myc [3], and term such reprogrammed cells as "induced pluripotent stem cells" or iPSCs. Nevertheless, techniques of reprogramming somatic cells are necessary to be nonviral, nononcogenic, and nongenetic modification before iPSCs can be used for treatment of human patients [24].

On the other hand, hESCs and their differentiated derivatives can be used in screening assays for the development of new potential pharmaceuticals and toxic as well as mutagenic compounds. While primary cell cultures or established cell lines are commonly used for both purposes, hESCs offer several advantages. hESCs have the ability to differentiate and efficiently produce unlimited numbers of cells representative of the three germ layers of embryos. The developmental equivalence of hESC-derived populations provides a more rigorous system for evaluating the teratogenic and embryotoxic effects of a substance, in addition to general mutagenic and cytotoxic effects [25]. A protocol based on hESCs differentiation has been established and validated for use in toxicity testing [26]. Additionally, genetic modification enables the tailoring of hESC lines for specific purposes. For example, specific genes can be altered to increase sensitivity

to mutagens or drugs [27, 28], or tissue-specific reporter genes can be introduced to detect changes in gene expression induced by toxic chemicals or therapeutic agents [29].

Finally, understanding mammalian embryogenesis through analysis of the early embryo is complicated by a number of factors, including size, availability, and the complexity of the embryo and uterine environment. Since hESCs are precursors to all embryonic lineages, these cells allow tracing the history from the root to individual branches of the cell lineage tree in a simplified and controllable culture environment. System for differentiation of hESCs *in vitro* provides experimental models that can be used to augment *in vitro* studies of *in vivo* mammalian embryogenesis, promoting a greater understanding of genes and signaling pathways regulating developmental decisions. One concern is that cell culture does not have a complex cell and tissue interactions that are critical to embryonic induction at distinct developmental stages. These cellular interactions, however, can be largely recreated in culture in the future with combination of tissue engineering in order to reflect the *in vivo* environment, allowing the better system to study embryogenesis.

4. Neural Differentiation of Human Embryonic Stem Cells

Growing evidence from animal experiments has shown that the formation of the nervous system can be induced by signals that emanate from a region of the embryo known as the "organizer" which secretes several molecules containing a direct neural activity, including noggin, chordin, and follistatin. These molecules act as central inhibitors of the bone morphogenetic protein (BMP) signaling pathway, a conserved inhibitory mechanism to neurogenesis from arthropods to vertebrates [30]. BMP antagonism has been recognized as the key and initiating process in neural induction and neuroepithelial specification and this is believed to occur as *a default pathway* [31, 32]. Based on this fundamental, hESCs have been efficiently induced to neural progenitor cells by applying the BMP inhibitor, noggin, into the culture system [33, 34]. Nevertheless, other findings challenge this model and suggest that some additional factors, including fibroblast growth factors (FGFs) and Notch, also participate in neural induction process. Aberration of FGF signaling diminished neural induction process from mouse ESCs [35], and the defect of FGF signaling was shown to have interconnection with BMP pathway to prevent neural differentiation of stem cells [30]. Similarly, Notch signaling plays an important function for neural fate entry of ESCs [36]. Constitutive activation of Notch signaling in mouse ESCs does not alter their phenotypes but promotes neural differentiation upon withdrawal self-renewal stimuli. In contrast, inhibition of Notch signaling suppresses the neural fate commitment. However, it was suggested that Notch signaling which induces neural differentiation requires parallel signaling through FGF pathway [37]. For this reason, a balanced view of neural induction process most likely demands incorporating both instructive and inhibitory signals.

Several strategies have been employed to achieve *in vitro* neural differentiation from hESCs, aiming at producing region specific neural progenitor cells or mature neuron/glial subtypes [38–40]. This was primarily accomplished by cell aggregation or embryoid body (EB) formation in neural induction medium and highly purified populations of neural progenitor cells could be further isolated and cultured [41, 42]. These neural progenitor cells could be expanded for over 25 population doublings as neurospheres in suspension culture. The neurospheres express markers of neuroectoderm, including Nestin, polysialylated (PSA) N-CAM, Musashi1, and PAX6 [41]. Importantly, the neural progenitor cells can differentiate into all derivatives of the nervous system, which are neurons, astrocytes, and oligodendrocytes. Importantly, EB-based protocol could induce specific neuronal subtypes, for instance, forebrain cholinergic neurons, which showed mature and functional electrophysiological profile [43]. However, since hESCs are pluripotent, neural differentiation via EB culture contains several limitations. Firstly, because of the cluster nature of EB, it is difficult to visualize the continual change of cell morphology in response to treatment. Next, the efficiency of neural conversion by EB formation is limited and neural lineage selection is necessary to ensure the enrichment of neural progenitor population. Besides, the structure of hESC aggregates prevents uniformly distribution of supplemented morphogens or growth factors. A high concentration of morphogens or growth factors is needed in order for the factors to diffuse inside the cell aggregates [44]. Therefore, cells on the surface of EB and those inside the aggregates will encounter a varied gradient of morphogens. And, due to this reason, a wide range of cell fates or cells at distinct developmental stages are derived from neurospheres. To overcome the limitations of EB protocol, a simpler way to reconstitute neural differentiation and achieve high efficiency of neural progenitor cell production is based on monolayer differentiation system of hESCs. It was noted that when applying similar monolayer differentiation system used for directing mouse ESCs to neural fate, hESCs became a large proportion of nonneural lineage cells. This mainly results from the highly active BMP signaling pathway in hESCs [7]. Thereafter, the success approach, showing to induce efficient neural conversion of hESCs, is by directly inhibiting the BMP/SMAD signaling [33, 34]. Supplementation of hESCs with noggin, a BMP antagonist, in neural inducing medium generated a highly pure and morphologically distinct population of cells that expressed several neural progenitor cell markers, including PAX6, Musashi1, and SOX2, without the detection of mesodermal and endodermal lineage markers [33]. To reinforce the purity of desired neural progenitor cells, the use of neural specific regulatory element to control expression of fluorescent protein is a powerful alternative tool for efficient identifying and isolating of hESC-derived neural progenitor cells by fluorescence cell sorting technique [45].

5. Human Embryonic Stem Cells as a Model of Human Neural Development

The embryonic origin of the brain is ectoderm. During neurulation, the neural plate folds over on itself and becomes

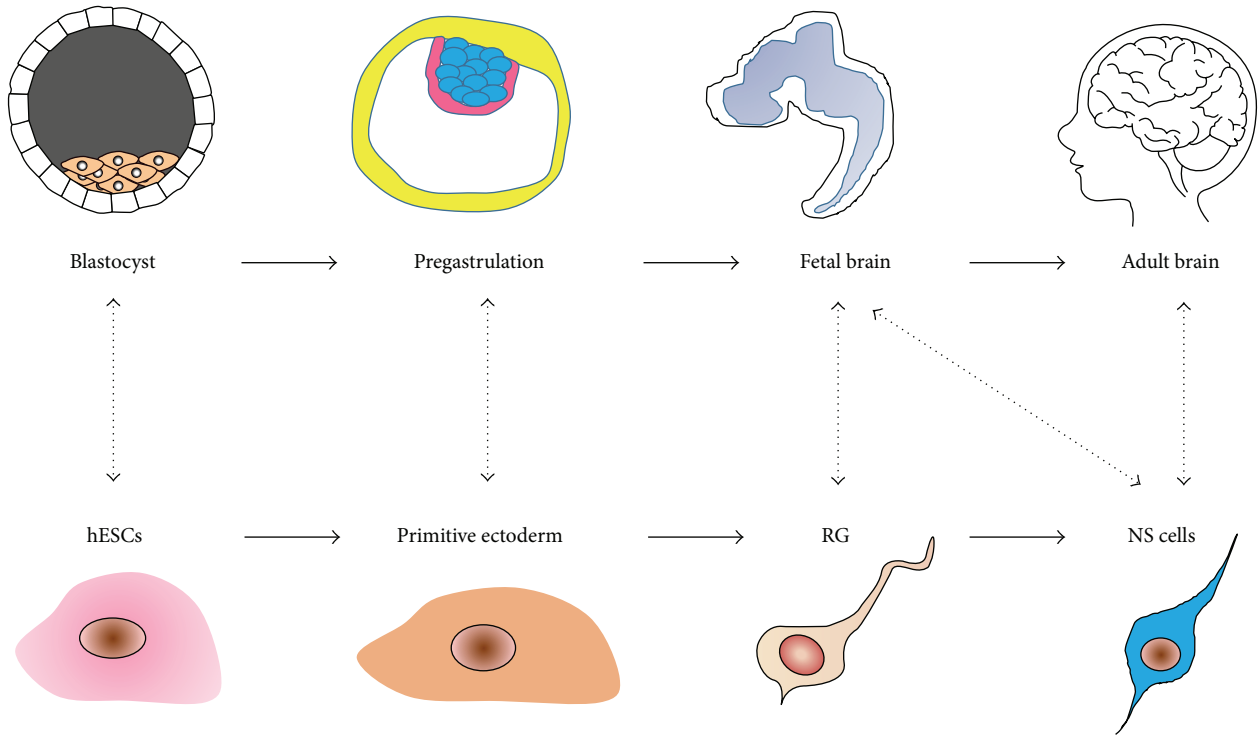

Figure 1: Developmental links between the different stages of neural derivatives of hESCs and their *in vivo* counterparts. Neural derivatives exhibit several similar characteristics to *in vivo* counterparts. The corresponding *in vivo* developmental stages are indicated and matched with the *in vitro* populations.

the neural tube. Consequently, the forebrain, midbrain, and hindbrain of the central nervous system are patterned and formed. Cell fate determination within the developing brain is controlled by signaling molecules, secreted by neighboring tissues. The development of animal models for neurological disorders is challenging and often questioned whether it fully recapitulates the human phenotypes. hESCs offer an alternative approach, because neural cells differentiated *in vitro* from hESCs display several properties equivalent to the developing embryonic brain [46]. As mentioned above, at least two systems have been well developed to explore human neurodevelopment from hESCs, which are cell aggregation and cell adherent culture system [42, 47]. Prominently, directed differentiation of hESCs in an adherent system shows remarkable similarity between *in vitro* differentiation and *in vivo* neuroectodermal development (Figure 1). The morphology of hESCs converts to columnar neuroepithelium after 7–10 days of the differentiation, which further develop into neural tube-like rosette structure at days 14–17 of the differentiation [42]. Because hESCs are generally derived from 5-6 day-old blastocyst embryos, generation of columnar neuroepithelium at day 10 of the differentiation, and formation of neural rosettes at days 14–17 correspond gastrulation phase at the start of the third week, later, and the establishment of the neural tube at the end of the third gestation week of a human embryo [48], respectively. After the completion of the neural plate development *in vivo*, the generation of neural tube will successively begin but will not take place homogeneously and synchronize throughout the developing neural tube. Instead, the neural tube is patterned to dorsoventral and rostrocaudal domains in order to set a grid-like structure of positional cues along its axes [49]. This emphasizes the critical need to establish positional information that could efficiently facilitate the generation of particular subtypes of neuron and glia cells *in vitro* from hESCs. To simulate the positional instruction in a laboratory culture, morphogens or growth factors that affect dorsoventral and rostrocaudal fate choices could be applied at the same time or in a sequential manner. By applying FGF8, which is known to influence mid-hindbrain neuron phenotype, and sonic hedgehog (SHH), a ventralizing factor, further prime hESC-derived neural progenitor cells into midbrain dopaminergic neurons [50]. Absence of these positional factors in the *in vitro* differentiation leads to the production of heterogeneous neuronal subtypes. This suggests that the supplementation of a specific set of morphogens at a specific time point is essential to pattern neural progenitor cells into a desired neuronal subtype [15, 51].

Formation of "neural rosette structure" is a morphological hallmark of an *in vitro* differentiation of hESCs to neural lineage, which mimic the *in vivo* structure of developing neural tube [48]. The culture of hESCs in chemically defined medium with BMP inhibitor, noggin, resulted in the generation of $PAX6^+/SOX1^-$ neural rosettes and succeeding supplementation of FGF2 induced $PAX6^+/SOX1^+$ neural progenitor cells [52]. Rosette-forming neural progenitor cells that express forebrain markers, such as Forse1, have presented the broadest differentiation potential, compared to other

neural progenitor cell populations [53]. These cells can be propagated in the presence of FGF2 and retained high Forse1 expression level, although FGF2 was recognized as caudalizing factor of neural progenitor cells [39]. Besides, the cells in neural rosettes are able to multiply by symmetric cell division and are capable of differentiating into cell types of both anterior-posterior, central-peripheral neuronal subtypes of the nervous system and are stable in a long term culture by stimulating SHH and Notch signaling pathways [53]. hESC-derived neurons can also be used to study synaptogenesis when plated onto specific feeder cells [54]. In addition to functional neurons, hESCs-derived neural progenitor cells are also able to produce astrocytes and oligodendrocytes either under basal conditions or instructive culture system, which is medium supplemented with ciliary neurotrophic factor or platelet-derived growth factor (PDGF) [55]. It is accepted that during early neurodevelopment, glial cells, including astrocytes and oligodendrocytes, are presented after the emergence of most neuronal cell types [56]. The similar scenario of neurogenesis to gliogenesis transition is conserved when explanted neuroectodermal cells are cultured or hESCs are differentiated along the neural lineage [14, 57]. Transcriptomics profile during neural differentiation of hESCs reveals distinct molecular features of multistage neural derivatives. The information obtained from this study might reflect mechanisms underlying brain development of human embryos [14]. The temporal changes of neuronal and glial differentiation of hESC-derived neural progenitor cells noteworthy reminiscent the timeframe observed from samples of embryonic tissues. This is suggested that the intrinsic program governing neuronal and glial lineage development is retained for hESC differentiation. Differentiated astrocytes, a robust derivative of hESC-derived neural progenitor cells, commonly express specific astroglial markers, including glial fibrillary acidic protein (GFAP) and S100β; however, oligodendrocytes are considered as a rare population obtained from hESC-derived neural progenitor cells [42]. It has been demonstrated that OlLIG2-positive neural progenitor cells can be readily obtained from hESCs in response to the treatment of SHH and RA [58]. These OLIG2-positive progenitor cells generate majorly motor neurons during neurogenesis; however, OLIG2-positive progenitor cells remain after neurogenic period and become mature oligodendrocytes. This suggests that the OLIG2-positive neural progenitor cells can give rise to oligodendrocytes and highlights the importance of OLIG2 in oligodendrocyte development *in vivo* [59].

6. The Approach to Model Neurodevelopmental Disorders by Human Embryonic Stem Cells

Neurodevelopmental disorders are caused by the impairment of the central nervous system during embryonic and early postnatal life. Early onset of neurodevelopmental disorders that are caused by genetic mutations could be probed by hESCs. This employs the advancement of preimplantation genetic diagnosis (PGD) during *in vitro* fertilization. Embryos diagnosed by PGD with congenital disease can be donated for research and cultured to the blastocyst stage for hESC derivation. To date, disease mechanisms underlying several neurological disorders have been approached by using diseased-specific hESCs.

Fragile X syndrome (FXS) is one of the most common cognitive disorders. It is caused by the mutation of FMR1 gene, encoding FMRP protein [60]. The FMR1 gene contains CGG repeats at 5′ upstream of the promoter region, and healthy individual caries this region up to 55 repeats. CGG repeats could expand during gametogenesis, and when it reaches 200 repeats will lead to FMR1 gene hypermethylation and gene silencing. FXS-hESCs were derived from PGD blastocysts and showed normal properties of human pluripotent stem cells [61]. Noteworthy, although FXS-hESCs contain 200–1,000 CGG repeats, FMR1 gene is unmethylated and FMRP is expressed normally. The silence of FMRP protein is found upon the differentiation of FXS-hESCs. Abnormal neural differentiation process was found in FXS-hESCs, compared to normal control hESCs [62]. The defects of neuron derived from FXS-hESCs included neuronal morphology, timing of development, and the aberrant expression of key neural lineage markers [62]. In FXS-hESCs, the neural progenitor cells mainly give rise to GFAP-positive glial cells, while the control hESCs became Tuj1-positive neurons. In addition, FXS-hESC neurons reduced the frequency and amplitude of their action potential, as well as spontaneous synaptic activity.

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder caused by the expansion of a CAG repeat in HTT gene. HTT gene encodes an amino-terminal stretch of polyglutamines, called huntingtin protein (HTT). HD is characterized by motor, cognitive, and psychiatric abnormality, but the exact mechanisms show repeated HTT protein caused neuron degeneration is not yet clear. Notably, because of the exclusively monogenic character of HD, this enables the potential use of hESCs to model HD pathology and screen for drug candidates. Stable expression of mutant HTT protein was introduced into healthy hESCs [63]. Neurons derived from these hESC lines showed HTT aggregates and abundant cell death in the culture. This deleterious phenotype can be rescued by silencing mutant HTT expression [63]. Knockdown of another gene implicated in HD pathology, such as Rhes, was also shown to recover HD pathology in the mutant neurons [64]. HD-hESCs are able to recapitulate some of the dominant phenotypes found in animal models, permitting future study in a detailed human context.

Rett syndrome (RTT) is a monogenic X-linked neurodevelopmental disorder. Major RTT patients are affected by MeCP2 gene mutation and appear as autistic-like behavior, sensory defects, ataxia, and microcephaly. MeCP2 is a methyl CpG binding protein and acts as a global transcriptional repressor. By employing genome editing technology to introduce mutant MeCP2, isogenic RTT-hESCs were generated [65]. MeCP2-mutant neurons exhibited central molecular and cellular phenotypes of RTT, including morphology and physiological defects. Striking global gene expression was downregulated in MeCP2-mutant neurons, which reflected the significantly reduced protein synthesis and could be

rescued by pharmacological and genetic manipulations [65]. Besides, the size of neuronal nuclei fails to enlarge at a normal rate during neural differentiation, compared to control hESCs [66]. This is accompanied by a significant reduction of ribonucleotide incorporation as well as the reduced level of brain-derived neurotrophic factor (BDNF). Reintroduction of MeCP2 could recover the nuclear size phenotype and BDNF expression level, suggesting the roles and functions of MeCP2 in RTT pathology [66].

Lesch-Nyhan syndrome (LNS) is a rare X-linked neurological disorder. Mutation of HPRT1 gene, encoding the hypoxanthine-guanine phosphoribosyl transferase (HGPRT), is a causative of LNS. HGPRT is an enzyme important for the generation of purine nucleotide, and the insufficiency of HGPRT leads to the accumulation of uric acid in the blood. Mental retardation is emerged as a symptom of LNS. There was an attempt to use hESCs to model LNS pathology. Mutant HGPRT was introduced into wild-type hESCs by homologous recombination [67]. LNS-hESCs resented several phenotypes mimicking LNS pathology, in particular uric acid accumulation. Although several downstream targets of HPRT1 mutations were explored, neural differentiation of LNS-hESCs has not yet been performed.

Malignant gliomas are the most aggressive nervous tumor found in both children and adults. Somatic mutation of H3F3A gene was found in major glioma patients. H3F3A gene encodes the histone H3 variant H3.3 and results in a Lys 27-to-methionine change (H3.3K27M) [68]; however, the role of H3.3K27M mutation in glioma formation is not fully understood. hESC lines carried H3.3K27K were generated and differentiated into neural progenitor cells [69]. Neural progenitor cells derived from H3.3K27M hESCs loss p53 expression and PDGFRA inactivation, leading to neoplastic transformation. Transcriptomic profiling reveals a resetting of the transformed neural progenitor cells to a developmentally more primitive stem cell state. This change is in accordance with major modifications of histone marks at numbers of master regulator genes [69]. The neural derivatives of these hESCs can also be used to screen for compounds that prevent tumor cell growth.

Down syndrome (DS) is caused by a trisomy 21 or extra chromosome 21, which is one of neurodevelopmental disorders manifested with cognitive abnormality. DS often associates with amyloid accumulation of early-onset Alzheimer disease (EOAD). This could be due to extra copies of over 400 genes that locate on chromosome 21. The critical region, 21q22.1–q22.3, contains genes, encoded amyloid protein, which is important in neurodevelopment and neurodevelopmental disorders [70]. As a result, accumulation of amyloid plaque in the brain leads to cognitive decline as EOAD in DS patients [71]. Previous report showed that hESCs were inhibited to differentiate into NPCs by accumulating of amyloid-β (Aβ1–42), which could be explained by the requirement of nonamyloidogenic pathway for hESCs to enter neural lineage [72].

Autism spectrum disorder (ASD) is another important neurodevelopmental disorder, manifested by aberration of social interaction and communication, as well as repetitive behaviors. While specific causes of autism spectrum disorders have yet to be found, many risk factors have been identified in the research literatures that may contribute to their development, including genetic factors. The deletion of 16p11.2 region on chromosome 16 is one of a well-studied ASD causative [73]. Recently, hESCs were reported in ASD model by genome engineering on 16p11.2 locus. Interestingly, transcription activator-like effector nuclease (TALEN), which is an essential tool in genomic editing, was capable of directed differentiating and highlighting hESCs as suitable model to study ASD pathology [74].

Based on the aforementioned examples, it is proved that hESCs present as a suitable platform system to model neurological disorders. Their application to the understanding of the molecular pathology of brain diseases can be significant in both basic research and therapeutic purposes.

7. Future Perspectives and Challenges

The notion of differentiation process of hESCs recapitulating the temporal changes found *in vivo* development has become widely accepted, not only for the nervous system, but also for other cellular lineages [13, 75]. Several early-onset neurological disorders showed the success of disease modeling by using hESCs. Immature phenotypes of neurons derived from hESCs hinder the applications of modeling for late-onset diseases [76]. Late-onset diseases could also be modeled by this system by progerin-induced aging [77]. Noteworthy, hESC differentiation system contains several limitations like other systems. Although the differentiation of hESCs displays an early stage of disease development, detailed characterization of *in vitro* neural derivatives is necessary in order to validate their *in vivo* counterparts and verify the stage of disease ontogeny.

In addition to hESCs, induced pluripotent stem cells (iPSCs) and induced neurons (iNs) have been intensively focused and employed as a disease modeling system [78, 79]. iPSCs and iNs could be generated from somatic cells of diseased-specific patients. Thus, these cells serve as a novel platform to functionally study specific mutations [78]. However, application of iPSCs and iNs to model diseases is restricted by several reasons, in particular epigenetic barriers of starting reprogrammed cells. Diseases which are related to imprinting genes and epigenetic anomaly, such as Fragile X syndrome [80], Angelman syndrome [81], and Prader-Willi syndrome [82], seem to be incompletely reprogrammed and unable to reset their epigenetic memory [32], which means iPSC and iN technology needed further development in order to overcome these issues [83].

Another challenge is the development of efficient protocol to derive specific neural derivatives. Each neurological disorder is usually affected by particular neuronal subtypes. Alternatively, the relevant neuronal subtypes are also needed to be isolated by using specific neuron reporter genes in order to obtain a pure population for further analysis. It is noted that the *in vitro* differentiation system cannot provide a spatial organization which exists as precise cell-specific microdomains or niche within the embryo. The further

development of culture systems, combining with tissue engineering technology, will offer an improved microenvironment and increase differentiation efficiency of hESCs toward desired neuronal cell types. The use of hESC-derived neural derivatives to explore brain development and disease mechanisms is still in a developing phase, and when completed, this system will provide a tremendous promise for both scientists and clinicians.

Competing Interests

The authors declare no competing interests.

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Review Article

Organotypic Cultures as a Model to Study Adult Neurogenesis in CNS Disorders

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Neural regeneration resides in certain specific regions of adult CNS. Adult neurogenesis occurs throughout life, especially from the subgranular zone of hippocampus and the subventricular zone, and can be modulated in physiological and pathological conditions. Numerous techniques and animal models have been developed to demonstrate and observe neural regeneration but, in order to study the molecular and cellular mechanisms and to characterize multiple types of cell populations involved in the activation of neurogenesis and gliogenesis, investigators have to turn to *in vitro* models. Organotypic cultures best recapitulate the 3D organization of the CNS and can be explored taking advantage of many techniques. Here, we review the use of organotypic cultures as a reliable and well defined method to study the mechanisms of neurogenesis under normal and pathological conditions. As an example, we will focus on the possibilities these cultures offer to study the pathophysiology of diseases like Alzheimer disease, Parkinson's disease, and cerebral ischemia.

1. Introduction

The use of organotypic cultures in neuroscience research covers the experimental gap between the *in vitro* and *in vivo* models. It provides an opportunity to cultivate CNS tissue for weeks or months, giving open accessibility to complex cellular systems. Organotypic cultures are mainly prepared from P3–P10 animals (rats or mice) or, with some exception, from adult CNS tissues (e.g., [1]). Young postnatal animals already possess essential cytoarchitecture and are easy to handle with respect to the embryonic tissue, and nerve cells survive in the explants more than in adult slices. Nevertheless, the neurodegenerative diseases linked to ageing and adult brain present characteristics one cannot model with cultures from young animals. Particularly, young brain differs from the adult brain in terms of synaptic development and genetic and metabolic profiles. The first attempt to culture brain slices from adult rats and mouse failed because of their ability to reduce the thickness. Slices from young or perinatal animal can reduce their thickness from 350–400 mm to 100 mm after 1 or 2 weeks of incubation, while the mature adult slices nearly kept their thickness over a two-week cultivation period with consequent necrosis of the central cellular layers (see [1] for a review). Several technical clues were used to overcome and implement the technique to culture organotypic slices from adult. Progressive reduction of serum in the culture medium allowed increasing cell viability in 6–8-week-old mice cultures [2]. It is still unclear why complete withdrawal of serum resulted successfully in prolonging cell viability but one can speculate that negative effects of serum might be caused by excessive neurotrophic and energy resources [3].

The advantage of using organotypic cultures derives from their usefulness in experiments that require long-term survival, such as live recording [4, 5] or pharmacology (chronic drug application) as well as electrophysiology and optogenetics [6].

The first attempt to maintain CNS slice tissues in long-term cultures has been the "roller tube" technique [7]. This technique, finally characterized more in detail by Gähwiler and Knopfel [8, 9], was developed on the basis of experiences based on multitude of studies with explants culture [10].

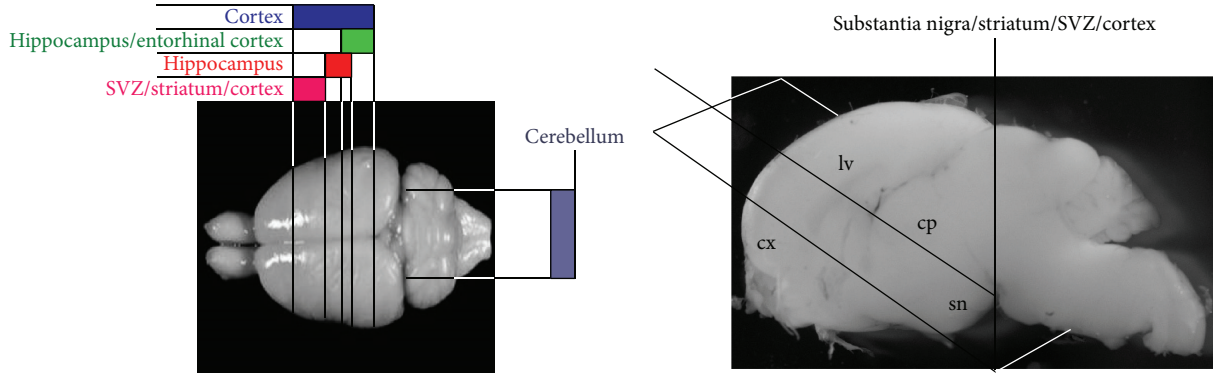

FIGURE 1: Different organotypic slices can be obtained from P2 to P7 postnatal forebrain or cerebellum by coronal sections of 350–400 μm (a). In (b), the brain sectioning to obtain 45° transversal sections of s. nigra/striatum/SVZ/cortex (sn, substantia nigra; cp, caudate putamen; lv, lateral ventricle; cx, cortex) used to model PD is shown.

In the roller tube cultures, the tissue is embedded in a plasma clot and attached on a glass coverslip. The coverslip with the embedded slice tissue is located in a tube that undergoes continuous slow rotation in a cell culture incubator. The oxygenation is maintained by continuous exchange of liquid-gas interface generated by the slow rotation. The technique was successively modified several times (e.g., [11, 12]), but the roller tube technique always yields very thin cultures (from an initial 400 μm to about 50 μm) with consequential preferential use for experiments that require optimal optical conditions (e.g., electron microscopy or electrophysiology).

At the beginning of the 90s, Stoppini and colleagues [13] published a new method to cultivate organotypic slices. In this method, brain slices were placed on a semiporous membrane and cultivated at the air-liquid interface. The absence of clot facilitates the studies of synaptic reorganization and became a useful tool to study plasticity and sprouting already during the first days of culture. The real advantage of this technique is that cultures are easily prepared and offer great advantages when a 3D structure is desired (from an initial 400 μm thickness, slices are cultivated up to 100–150 μm). As described more in detail below, the air-liquid interface has become a key instrument to study adult neurogenesis. Organotypic slices can be obtained from different brain regions (as described in Figure 1), but for the study of adult neurogenesis in normal and pathological conditions, the hippocampal region containing the SGZ and the slices containing the SVZ are most preferred. Thus, for example, neurogenesis in Alzheimer disease or Parkinson's disease can be studied in hippocampus/entorhinal cortex or s. nigra/striatum/SVZ/cortex slices [14, 15]. Organotypic cultures match the tridimensional space where neural progenitors migrate to reach maturation *in vivo*. In the paper by Vergni and colleagues [16], we ideally represented the slice culture comprising subventricular zone, as the spatial extension to elaborate a mathematical model to describe neuroblasts activation and migration following oxygen and glucose deprivation.

2. Adult Neurogenesis in CNS

Neurogenesis in the normal adult brain occurs mainly in the dentate gyrus from the subgranular zone of hippocampus (SGZ) and in the olfactory bulb from the subventricular zone of lateral ventricle (SVZ). In both cases, the neurogenic niches, the SGZ and SVZ, host multipotent cells that give rise to neuroblasts or glioblasts throughout life. The stem cells in the neurogenic niches (type 1 in the SGZ or B cells in the SVZ) give rise to the more proliferative transit-amplifying progenitors (type 2 or C cells) that in turn give rise to neuroblast (type 3 or A cells) or glioblasts [17]. The study of adult neurogenesis in the SGZ is relevant especially for the study of repair mechanism of neurodegeneration related to loss of memory or neuropsychiatric disorders (for a review see [18]). New neuroblasts generated in the SGZ migrate to the granular zone of the dentate gyrus covering short distances, whereas neuroblasts generated in the SVZ move through the rostral migratory stream (RMS) to the olfactory bulb (OB) where finally they migrate radially and differentiate into new neurons.

Adult neural stem cells (NSC) are a specialized form of glia, deriving from the embryonic radial glia [19]. They possess a regional pattern within the SVZ which is related to their embryonic origin and reflect the ability to generate different types of OB interneurons (e.g., granule cells, TH+, or calbindin+ periglomerular cells; see [20]). The neurogenic moiety is guaranteed by different subpopulation of multipotent cells characterized by the expression of different markers in different temporal stages, as shown in Table 1. NSC can be activated, and neurogenesis can be induced, under different conditions. A predominant role *in vivo* is covered by the blood vessel and local vascular plexus that bring trophic factors or stress molecule signals. This was recently demonstrated by Katsimpardi et al. [21], who potentiated the neurogenesis of old animals after transfusion of blood from younger animals. The absence of local circulation in the organotypic cultures is fixed by using different trophic factors in the medium that stimulate intrinsic signals, mainly

Table 1: SVZ neural stem cell characterization by cellular marker expression.

	GFAP	CD133	EGFR	Nestin	DCX	βIII tubulin	NeuN	PDGFR	Proliferation
B cells									
qNSC	+ + +	++	−	−	−	−	−	−	−
aNSC	+ + +	++	++	+	−	−	−	+	+
C cells									
Transit amplifying	+	−	+	+ + +	−	−	−	++	+ + +
A cells									
Neuroblast	−	−	−	+	+ + +	+	−	−	++
Immature neurons	−	−	−	−	−	+ + +	+	−	+
Glioblast	+ + +	−	−	+	−	−	−	+ + +	+

Proliferation is expressed by BrdU incorporation ability (Codega et al., 2014) [17]. Quiescent neural stem cells, qNSC, and activated neural stem cells, aNSC.

transcription factors, for example, sox2, olig2, or the bmp family [22, 23].

3. Techniques Used to Study Neurogenesis in OC

From a technical point of view, the organotypic cultures represent a versatile tool to study neurogenesis or cell regeneration. *In vivo* neurogenesis is a multistep process that involves proliferation, migration, and differentiation of neural stem cells as well as integration into preexisting network and functionality [24]. Each of the mentioned steps can be assayed in an organotypic slice. The method more used for studying the cell proliferation is the labelling with cell duplication markers. The most used ones are the nucleotide analogue 5-bromodeoxyuridine (BrdU) or 5-iododeoxyuridine (IdU) and the nuclear protein Ki67. BrdU and IdU incorporate into the duplicating DNA (during the S phase), whereas Ki67 protein is a nuclear protein expressed in all phases of cell duplication, all of which are subsequently visualized by immunofluorescence. In addition, the combination of BrdU and IdU can be used for time window experiments and cell characterization (for a review see [25]). Infection of fluorescent proteins with retroviruses is often used for time lapse experiments. Time set of different infections allowed the researcher to perform connectivity and lineage studies on newly generated cells directly *in vitro* [12, 26].

To study cell differentiation, BrdU/IdU immunofluorescence can be combined with antibodies for the different markers of differentiation shown in Table 1. Differentiation can be assayed also in organotypic cultures derived from transgenic animal expressing fluorescent reporter genes. This is the case of reporter mouse lines in which neural stem and progenitor cells express various fluorescent proteins (GFP, CFPnuc, H2B-GFP, DsRedTimer, and mCherry) under the control of the nestin promoter [27]. With these animals, one can follow all processes of differentiation and proliferation and evaluate the changes induced by various neurogenic and antineurogenic stimuli [28]. Using the fluorescent reporter genes is convenient also to verify the final integration of newly generated cells into a preexisting network by electrophysiology.

Organotypic cultures can be used especially to study the mechanisms of integration of new cells into preexisting circuitries. In the model proposed by Tønnesen et al. [29], they transplanted *in vitro* cultured GFP-TH neurospheres overexpressing embryonic Wnt5a into striatal organotypic slices. They observed neuronal differentiation (expression of neuronal markers and spontaneous firing of action potentials), synapse formation, and functional expression of dopamine D2 autoreceptors. In the same work, the authors could activate or inactivate optogenetically grafted cells demonstrating bidirectional synaptic interactions between grafted cells and host neurons and extensive synaptic connectivity within the graft.

4. Organotypic Cultures as a Model for CNS Neurodegeneration and Studies on Neurogenesis

4.1. Ischemia. Cerebral ischemia is generated by the loss of oxygen and nutrient in the brain. *In vivo* ischemia is generated in mice by occlusion of the middle cerebral artery that generates the damage of specific areas (cortex, striatum, and hippocampus), degeneration of neurons, and activation of microglia. In organotypic slices ischemic damage is modelled by oxygen and glucose deprivation (OGD). The neuronal damage generated is divided in a central core, where neurons die by necrosis, and a surrounding penumbra, where neurons die more slowly by apoptosis. This latter part sends death signals and starts a dual communication with the neurogenic niches. On one hand, the penumbra sends SOS signals to the SVZ which generates protective factors and activates neurogenesis [30]. On the other hand, death signals released from the focus of degeneration form a biochemical barrier impeding the neuroblasts migrating from SVZ to the damaged region. This was also modelled *in silico* by using cortex/SVZ/striatum cultures [16]. In this paper, we identified extracellular ATP and microglia activation as factors impeding neurogenesis and the interaction of SDF-1α with its receptor CXCR4 as a key signalling pathway driving neuroblasts migration.

The effect of neuroinflammation on neurogenesis after OGD has been largely studied in hippocampal organotypic

cultures. In this culture, OGD generates a selective damage in the CA1 region and microglia recruitment to the damaged zone. Several papers demonstrated that anti-inflammatory treatment in organotypic slices facilitated the neurogenesis from the SGZ through the inhibition of both the p38 mitogen-activated protein kinase and metalloproteinases [31, 32]. Metalloproteinases are involved in the activation of several neuroinflammatory events. Also during OGD, this class of enzyme can sustain neuroinflammation and modulate neurogenesis in the dentate gyrus. In fact, the modulation of culture microenvironment after OGD in hippocampal organotypic cultures can promote the proliferation of glioblasts with predominant generation of oligodendrocyte progenitors [32].

4.2. Parkinson's Disease. Parkinson's disease (PD) is caused by the selective neurodegeneration of dopaminergic neurons. The main affected areas are the substantia nigra (s. nigra) and striatum. Nevertheless, dopaminergic degeneration affects also other areas like cerebral cortex, globus pallidus, and thalamus. The lack of dopamine and an unbalance of dopaminergic and glutamatergic signal cause the classical motor symptoms of bradykinesia, rigidity, resting tremors, and loss of postural reflexes. The system "s. nigra/striatum/cortex" has been reproduced *in vitro* with different organotypic cultures. The first attempt was made by Plenz and Kitai [33]; they cocultivated with the roller tube technique the three main areas involved in PD degeneration: cerebral cortex, striatum, and s. nigra. After a few days, the three regions were connected by new tyrosine hydroxylase positive fibers (TH^+). Another coculture model to study dopaminergic degeneration was performed by combining the basal nucleus of Meynert, ventral mesencephalon, parietal cortex, and dorsal striatum of postnatal 7–9 rat pups [34]. Nevertheless, the presence of the SVZ in these organotypic slices is important beside the interest in adult neurogenesis. In fact, the SVZ is directly innervated by TH fibers suggesting that these can sustain the maintenance of the neurogenic niche. We described an organotypic model in which we maintained in one single slice the connection between s. nigra/striatum/cortex and SVZ [15]. In this culture, dopaminergic degeneration can be obtained either by mechanical lesion or by 6-OHDA injection. In both cases, the loss of dopamine stimulated the TH expression and proliferation of SVZ cells, suggesting the neurogenic niche can be activated following dopaminergic lesion. Moreover, while 6-OHDA generated more specific dopaminergic degeneration, the mechanical lesion can be used to study glutamatergic pathways or GABA-ergic degeneration which allows us to represent, respectively, early or advanced PD model (see [35] for a review).

4.3. Alzheimer's Disease. Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by a progressive loss of memory and dementia. The causes and aetiology of AD are still unknown and the brain regions more affected are the hippocampus and neocortex. At molecular level, brain patients are characterized by amyloid plaques (dense and insoluble deposits of the beta-amyloid peptide-Aβ) and neurofibrillary tangles (intracellular aggregates of the hyperphosphorylated microtubule-associated protein tau). One of the first pieces of evidence of neurodegeneration in AD is the neuronal loss of cholinergic fibers of the entorhinal cortex connecting the CA1 layer of hippocampus [36]. AD is mimicked by different animal models from invertebrate [37] to rodents [38]. Except for the mouse SAMP8 [39], all transgenic models combine different mutations for amyloid precursor protein, tau, and presenilin 1.

The best organotypic model that represents cellular alteration observed in AD is the hippocampal slice. Alberdi and colleagues [14] used an organotypic slice in which they maintained the connection between entorhinal cortex and hippocampus. This model could be particularly useful to study cholinergic degeneration and activation of neurogenesis in the SGZ. Induction of neurodegeneration is obtained by treatment of slices with different Aβ peptides. As for several animal models, Aβ together with neuronal damage may play a role in the regulation of adult neurogenesis [4]. In particular, low concentration and the small $A\beta_{25-35}$ peptide result in an increase of mossy fibers density and stimulation of endogenous SGZ neurogenesis. Nonetheless, neuroinflammation associated with Aβ toxicity can mediate the inhibition of SGZ neurogenesis through the release of various proinflammatory cytokines.

5. Other Organotypic Models for Neuroregeneration Studies

In addition to neurogenesis in the brain, organotypic cultures were employed also to study cell regeneration in spinal cord and PNS. Organotypic slices of mice adult spinal cord can be prepared with the liquid-air interface [40]. Spinal cord cells incorporate BrdU and express nestin, Oct3/4, and Dppa in the inner mass and have been used for modelling regeneration in spinal cord lesion [41]. Enteric neural stem cells (ENSC) have been isolated from adult intestine. They express markers like Ret, p75, and CD49b [42] and can differentiate primarily into glia [43] but also into neurons and myofibroblasts. If neurogenic potential of ENSC has been demonstrated *in vivo*, there is still discrepancy in results obtained *in vitro*. *Ex vivo* organotypic cultures from longitudinal muscle and myenteric plexus tissue demonstrated 5-ethynyl-2′-deoxyuridine incorporation in ENSC and potential proliferation dependent on the PTEN/PI3K/Akt pathway [44]. To investigate the neurogenesis in auditory system, Aburto and colleagues [45] used organotypic cultures of explanted chicken otic vesicles (OV). Neuroepithelial otic progenitors transit through states of cell proliferation, cell fate specification, cell cycle exit, migration, and differentiation showing characteristics of neural stem cells. In this case, autophagy covers a relevant role in maintaining clearance of apoptotic cells and facilitating neuronal differentiation of progenitor cells [45]. The inhibition of LC3B, a gene marker of autophagy, in organotypic cultures of OV provoked the misregulation of the cell cycle and impairing neurogenesis.

Other factors like GDNF can contribute to the final neuritogenesis of NSC-derived PNS neurons as demonstrated in organotypic slices of mice spiral ganglia [46]. Stimulation of GDNF-family receptor α-1 (GFRα1) activated two parallel

pathways, PI3K/Akt and MEK/Erk especially during early postnatal days.

6. Concluding Remarks

Organotypic cultures of the neurogenic niches represent a valid alternative method to study neurogenesis in the CNS, which complements *in vivo* models and neurosphere cultures. Moreover, these cultures represent a good method to model brain damage in diseases like AD, PD, or stroke and to study how to implement neurogenesis as a potential mechanism of brain repair in these disorders. Organotypic slices have been used especially for toxicity studies; therefore, in neurogenesis research, they became valuable for testing drug effects on neurogenesis activation or improving cell fate specification.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Neural Conversion and Patterning of Human Pluripotent Stem Cells: A Developmental Perspective

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Since the reprogramming of adult human terminally differentiated somatic cells into induced pluripotent stem cells (hiPSCs) became a reality in 2007, only eight years have passed. Yet over this relatively short period, myriad experiments have revolutionized previous stem cell dogmata. The tremendous promise of hiPSC technology for regenerative medicine has fuelled rising expectations from both the public and scientific communities alike. In order to effectively harness hiPSCs to uncover fundamental mechanisms of disease, it is imperative to first understand the developmental neurobiology underpinning their lineage restriction choices in order to predictably manipulate cell fate to desired derivatives. Significant progress in developmental biology provides an invaluable resource for rationalising directed differentiation of hiPSCs to cellular derivatives of the nervous system. In this paper we begin by reviewing core developmental concepts underlying neural induction in order to provide context for how such insights have guided reductionist in vitro models of neural conversion from hiPSCs. We then discuss early factors relevant in neural patterning, again drawing upon crucial knowledge gained from developmental neurobiological studies. We conclude by discussing open questions relating to these concepts and how their resolution might serve to strengthen the promise of pluripotent stem cells in regenerative medicine.

1. The Developmental Origins of the Nervous System: An Overview

The process of neurodevelopment is spatiotemporally regulated and necessitates sequential, progressive restrictions in cell fate. Although some interspecies differences in both cytoarchitecture and molecular machinery do exist between mouse and man, rodent models have illuminated key underlying mechanisms of lineage restriction to a variety of cell types. These insights have provided invaluable guidance for the predictable manipulation of human pluripotent stem cells (hPSCs) into myriad cell fates. From the point of fertilisation of the secondary oocyte, cells commence asymmetric division and sequentially give rise to the 2-, 4-, and then 8-cell stage blastomere, which subsequently develops into the blastocyst (Figure 1). Oct3/4 serves to maintain pluripotency in the inner cell mass (ICM) of the blastocyst. Although interspecies differences in cell-type specific factors exist, ultimately and following implantation and gastrulation, 3 distinct germ layers emerge: endoderm (which forms the lining of internal organs), mesoderm (which gives rise to bone, muscle, and vasculature), and ectoderm (from which results the nervous system and skin). Figures 1 and 2(a) describe developmental processes involved in specification of the 3 germ layers. During gastrulation, this 3-layered structure undergoes progressive and stereotyped morphological transformations. The mesoderm and endoderm invaginate inwards and the ectoderm forms an epithelial sheet which ensheathes a central cavity. The region of the ectoderm surrounding the neural plate becomes epidermis (Figure 2(a)). An important aspect of embryogenesis is the assignment of developmental axes. "Anterior-posterior" can be used to refer to the proximal-distal axis, which is based on proximity to the future placenta (in the early blastocyst the proximal pole is represented by

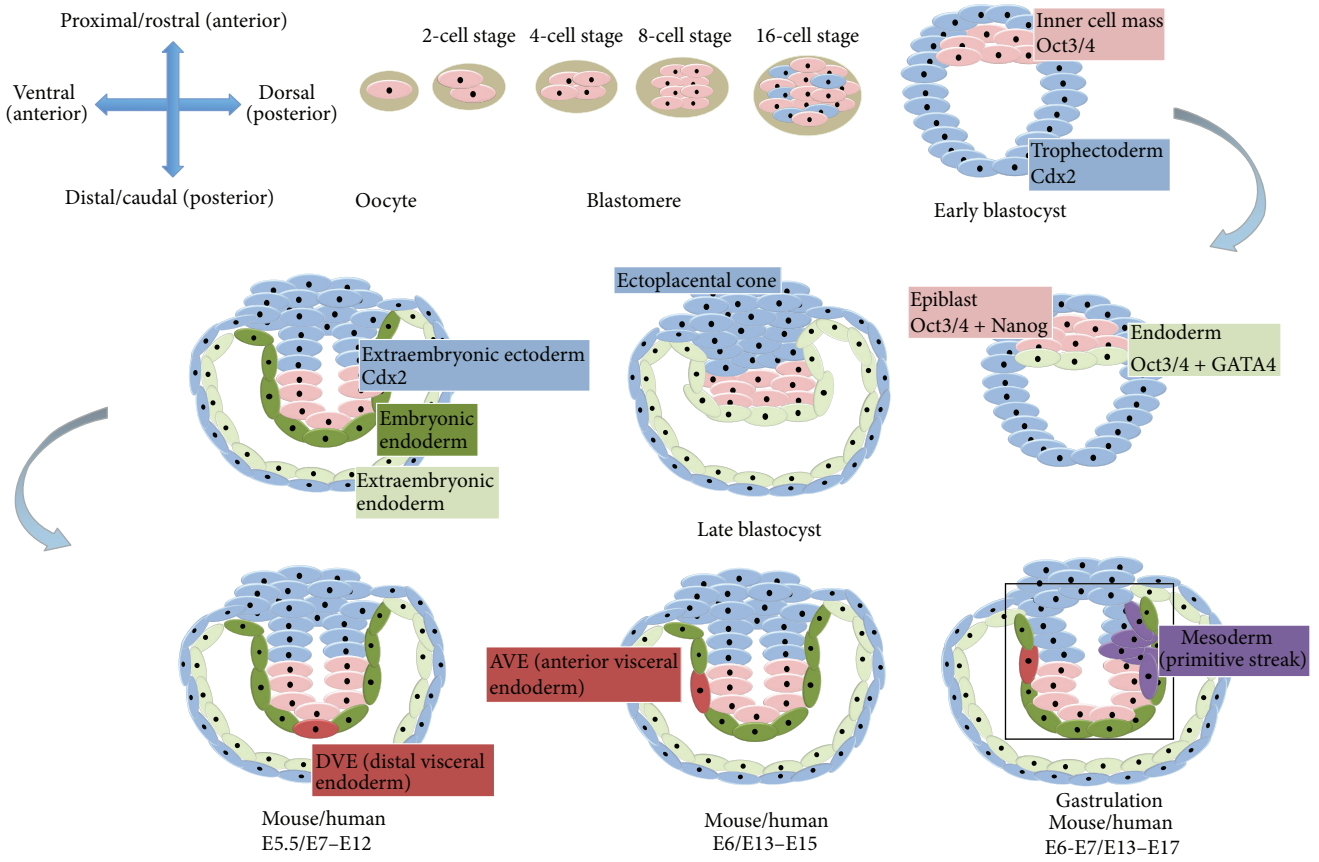

Figure 1: Developmental stages of mouse embryo. First row (left to right), from the secondary oocyte the blastomere develops (2-cell, 4-cell, 8-cell, and 16-cell stages) to give rise to the early blastocyst formed of trophectoderm (cells that express Cdx2) and inner cell mass cells (that express Oct3/4). Later, the inner cell mass gives rise to the epiblast (cells that express Oct3/4 and Nanog) and endoderm (expressing Oct3/4 and GATA4). Second row (right to left), in the late mouse blastocyst Cdx2 positive cells give rise to the extraembryonic ectoderm and ectoplacental cone. At the same time the endoderm divides into an embryonic endoderm and an extraembryonic endoderm. The epiblast and the extraembryonic ectoderm form a cavity lined by embryonic endoderm. From the embryonic endoderm the distal visceral endoderm is formed (DVE). Third row (left to right), the DVE migrates proximally and will be known as the anterior visceral endoderm (AVE). The final image (third row, right) shows the development of the primitive streak (mesodermal cells) at the opposite (posterior) pole from the AVE. N.B. There are 2 different types of endoderm called extraembryonic and embryonic; these differ in their potency and give rise to distinct cellular derivatives. All timelines are given for mouse and human embryonic development.

the ectoplacental cone as depicted in Figure 1). Later, the proximal-distal axis will become the future rostrocaudal axis in vertebrates. However, the term "anterior-posterior axis" can also sometimes refer to the dorsoventral axis in the adult state, a distinction that is primarily based on position of the abdomen (ventral) as opposed to the back/spinal column (dorsal). Therefore, for ease of reference this review will use the terms rostrocaudal ("R-C") and dorsoventral ("D-V") axes.

Three principal events characterise early neurodevelopment. First, the process of *neural induction* specifies a region of the embryonic ectoderm to form the neural plate (Figure 2(a) [1]). Second, a process termed *neurulation* occurs through serial morphological transformations to give rise to the neural tube (Figure 2(b); [2]). This process consequently imparts further histological architecture to the developing neuraxis. Third, the neural tube is divided into functionally and spatially distinct regions by a programme of inductive interactions called *neural patterning* (Figure 2(c) [3]). In humans, neurulation occurs at 21 days after conception and depends on a precise sequence of changes in the three-dimensional shape of individual cells including changes in cell-cell adhesion. Specific gene expression profiles are controlled by neuraxial position and local extrinsic morphogenetic instruction. Gastrulation leads to the formation of the notochord, a distinct cylinder of mesodermal cells extending along the midline. Ectoderm lies adjacent to the notochord, from which it receives inductive signals to form neuroectoderm. Neuroepithelium of the neural plate then undergoes complex morphogenetic movements involving cell division, morphological changes, and migration to permit neural tube formation. Following neural tube closure, the dorsomedial borders of the neural folds become neural crest derivatives. Cell movements at this stage are critical in producing different neuraxial regions. For example, in the ventral midline of the neural tube, cells become a specialized

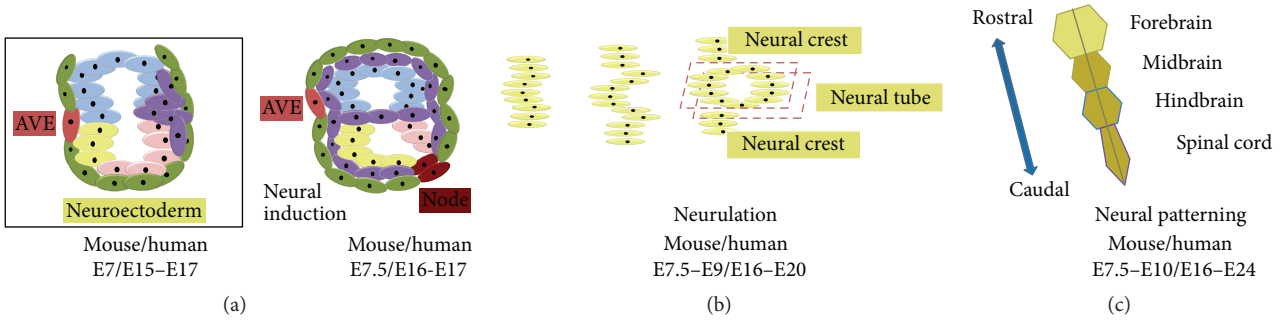

Figure 2: Neural induction, neurulation, and neural patterning overview. (a) Neural induction: neuroectoderm (neural plate) differentiation happens under the influence of the AVE. The mesodermal cells start migrating in all directions and envelop the embryo between the endoderm and the ectoderm. At the distal pole of the embryo the node develops, to further act as the "trunk organiser." (b) Neurulation: from the neural plate, cells start to proliferate and invaginate in order to form the neural tube and neural crest which derives from the dorsomedial borders of the neural folds. (c) Neural patterning: cells from the neural tube start to differentiate into precursors for forebrain, midbrain, hindbrain, and spinal cord according to a rostrocaudal axis. All timelines are given for mouse and human embryonic development.

region called the floor plate (Figure 4(d)). A large variety of distinct neuronal subtypes are generated during mammalian neurodevelopment. This diversity is an absolute prerequisite for the establishment of functional neuronal circuits.

In summary, the consecutive steps of neurodevelopment include neural induction from embryonic ectoderm, patterning along rostrocaudal (R-C), and dorsoventral (D-V) axes (allowing regionally determined functional heterogeneity) and subsequently terminal differentiation into diverse postmitotic neuronal subtypes [2]. Such insights from developmental neurobiology provide a conceptual framework for the directed differentiation of hPSCs and allow experimental interrogation of the molecular "logic" of neuronal subtype diversification [4]. Taken together with the understanding that region, and/or subtype, specific degeneration of neurons underpin the majority of neurodegenerative diseases, these facts provide a compelling rationale to predictably manipulate the cell fate of hPSCs in order to generate clinically relevant populations of region specific neurons and glia for further study [5].

2. Neural Induction

The first mechanistic insights into neural induction originate from seminal experiments by Spemann and Mangold in the early part of the twentieth century. In these studies, dorsal mesoderm was transplanted into the ventral embryo and generated a secondary host-derived neural tube. The graft itself was found to contribute to secondary mesodermal structures including the notochord, while the neural tissue was host-derived. The ability of the dorsal blastopore lip to reprogram surrounding tissues when transplanted ectopically justifies its designation as "organiser tissue." Equivalent organiser regions in other vertebrates were subsequently discovered by the elegant work of Waddington in the 1930s, including "Hensen's node" in birds and mammals (Figure 2(a)). Organiser tissue's capacity to precipitate ectopic neural induction *interspecies* suggests evolutionary conservation of underlying mechanisms. The notion of inductive signals orchestrating the process of neural induction has become widely accepted. Accumulating evidence suggests a spatiotemporal interdependence of several signalling pathways in neural induction, which somewhat challenges the concept of organiser tissue. The molecular pathways underlying neural induction remained elusive until the 1990s, when *Xenopus* studies first reported that transient dissociation of gastrula-stage animal caps into single cells resulted in neural fate acquisition and that misexpression of a dominant-negative Activin receptor, since being discovered to inhibit multiple transforming growth factor (TGFβ-) related factors, ectopically generated neural tissue at the expense of mesoderm specification. These studies suggest that neural induction may occur through a "de-repression" strategy (i.e., the removal of an inhibitory signal). Figure 3 depicts the relevant pathways in this process.

2.1. The Role of TGF-β Signalling Superfamily Members in Neural Induction. The molecular machinery of TGF-β signalling is relatively well understood: ligand binding causes receptor dimerization and initiates a signal transduction pathway and activates a family of cytoplasmic proteins, the Smads, by phosphorylation. Eight Smad proteins are encoded in the human genome, although only five of these (Smad 1, Smad 2, Smad 3, Smad 5, and Smad 8) act as substrates for the TGF receptor family; these are commonly referred to as "receptor-regulated Smads," or just "RSmads." Broadly, the TGF-β signalling superfamily encompasses both the Activin/Nodal and bone morphogenetic protein (BMP) signalling pathways [6]. The substrates for BMP signalling are Smads 1, 5, and 8, while the Activin/Nodal receptors activate Smads 2 and 3. Co-Smad (Smad 4) functions as a common partner for all RSmads, whereas Smad 6 and Smad 7 are inhibitory. Smad/Smad 4 complexes translocate to the nucleus and activate gene expression.

2.1.1. BMP Antagonism. In the early 1990s Noggin, Follistatin, and Chordin were identified as genes encoding proteins with neuralizing activity that were expressed in organiser tissue. These proteins are inhibitors of BMP signalling, with a particular bias towards antagonising BMP4, an inhibitor

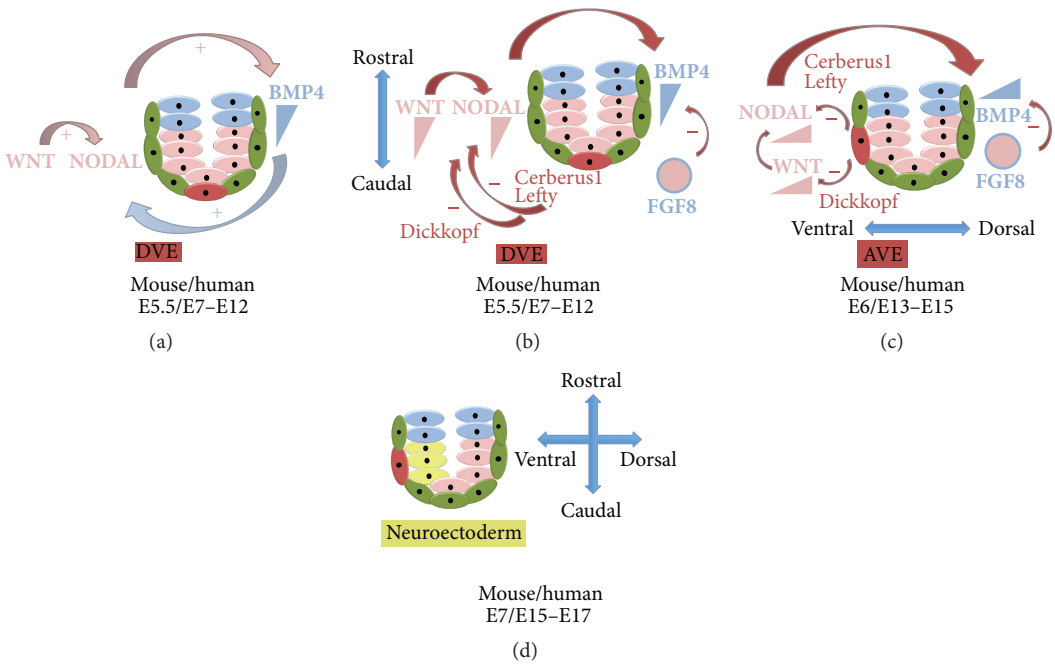

Figure 3: Molecular pathways in neural induction. (a) The epiblast (depicted in pink) expresses Nodal. The epiblast through Nodal stimulates (pink arrow) the expression of BMP4 (depicted in blue) in the extraembryonic ectoderm (blue cells). The extraembryonic ectoderm, by the action of BMP4, stimulates (blue arrow) the WNT (depicted in pink) pathway in the epiblast that in turn further activates (pink arrow) Nodal expression. Thus, there is a positive feedback loop between Nodal, BMP, and WNT. Colour scheme: arrows corresponds to the related tissue/morphogen. (b) The DVE (depicted in red) expresses Cerberus1 and Lefty (also depicted in red) to inhibit Nodal expression, therefore downregulating Nodal in its proximity. It also expresses Dickkopf (depicted in red), a protein that inhibits WNT3 signals close to the DVE. Downregulating Nodal and WNT also inhibits BMP4 expression close to the DVE. Thus, there is a gradient of Nodal, WNT, and BMP with a high expression rostrally and low expression caudally. FGF8 (pink and blue), expressed both in the epiblast (pink) and extraembryonic ectoderm (blue), also inhibits BMP4 contributing to the gradient. Colour scheme: arrows corresponds to the secreted inhibitory molecules/tissue source (DVE). They show the consequence of the negative feedback that creates the morphogen gradients in the R-C axis. (c) The DVE migrates into the AVE and the gradients are thus remodelled with low Nodal, WNT, and BMP expression ventrally and high dorsally. (d) Due to these gradients the neuroectoderm is formed at the ventral pole of the epiblast. Colour scheme: arrows corresponds to the secreted inhibitory molecules/tissue source (AVE). They show the consequence of the negative feedback that creates the morphogen gradients in the D-V axis. All timelines are given for mouse and human embryonic development.

of neural fate. BMP4 is expressed widely at the onset of gastrulation (Figure 3(a)) but is subsequently downregulated in the neural plate following the emergence of the organiser region (Figure 2(b)). Blockade of BMP signalling leads to an expanded neural plate in whole embryos, while mice with null mutations in BMP antagonists (such as Noggin and Chordin) show a significantly reduced brain size [1]. The wider roles of BMP pathway in embryo development are comprehensively reviewed elsewhere [7].

These facts, taken together, allow a simple molecular pathway for neural induction to be considered: the extraembryonic ectoderm produces BMPs to promote epidermal differentiation, while neural inducing regions (organiser tissues) antagonize BMPs to permit neural induction (Figures 3(a)–3(d)). This can be achieved by blocking BMP mRNA at the pregastrula stage by Fibroblast Growth Factor (FGF). Alternatively, the BMP protein can be antagonised at the gastrula stage by aforementioned factors secreted from organiser regions. Against this background, the "default model" of neural induction was formulated, hypothesizing that gastrula-stage ectodermal cells have an autonomous predilection to differentiate into neural tissue and that this process is inhibited by BMPs. In contrast to this model, subsequent studies have demonstrated that organiser tissue/BMP antagonism can be dispensable for neural induction, suggesting that additional mechanisms/signalling pathways merit consideration in this review, given their potential significance in informing strategies for neural conversion of hPSCs [1, 8, 9].

2.1.2. Activin/Nodal Antagonism. A significant majority of studies have focused on the role of BMP inhibition in neural induction during vertebrate development. However, the importance of other members of the TGF-β superfamily, including Nodal, is also well established [10]. Nodal acts as an inhibitor of neural induction [11], while Nodal knockout embryos show increased neuroectoderm specification [12]. A role for Nodal inhibition in neural induction from mouse and human embryonic stem cells (ESCs) is well established, both alone [13–15] and combinatorially with BMP antagonism [16]. Nodal is expressed throughout the epiblast (Figure 3(a)) and inhibitors of this pathway have been identified in the DVE/AVE [17], which play crucial regulatory roles both in

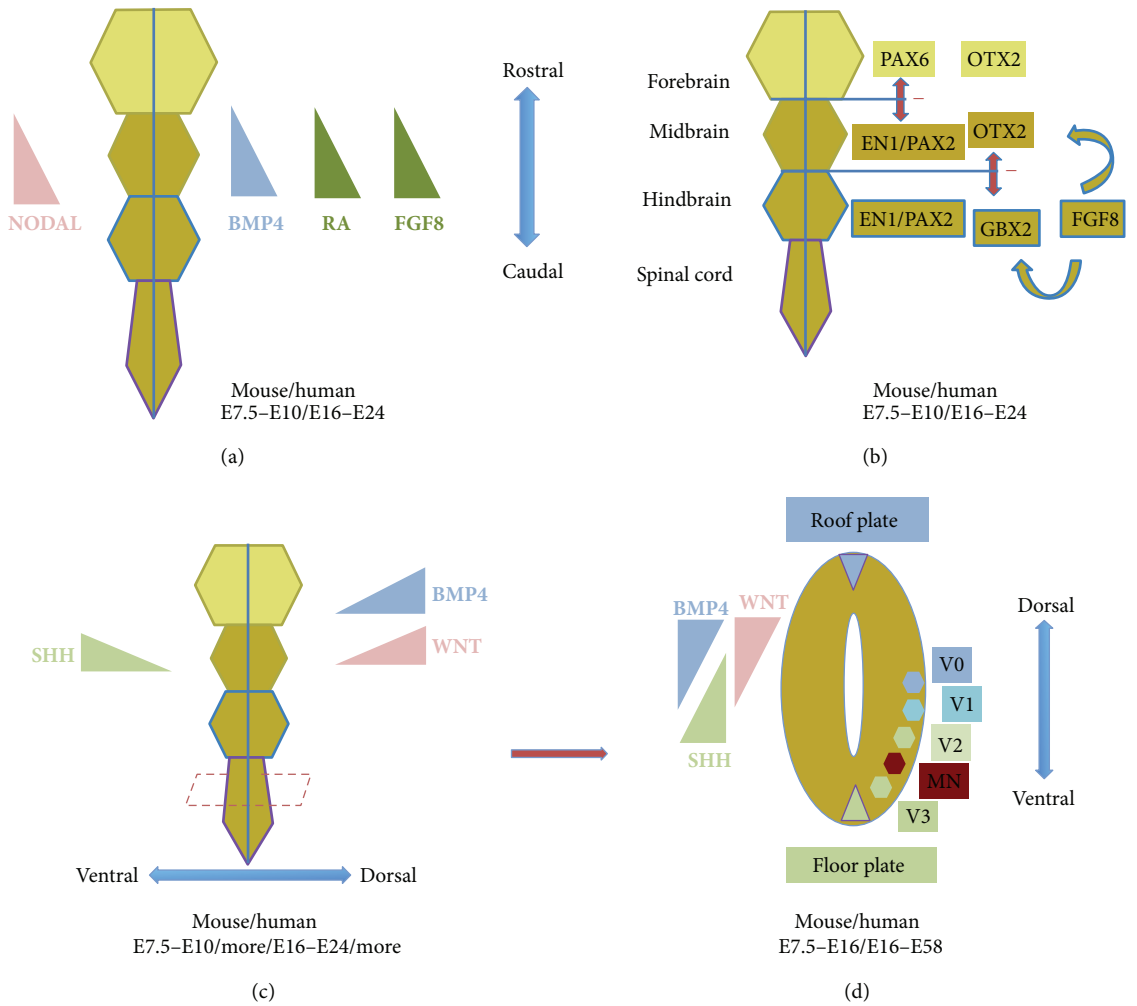

Figure 4: Neural patterning. (a) Rostrocaudal gradients of Nodal, BMP4, RA (retinoic acid), and FGF8 important in rostrocaudal patterning. (b) The interplay between different factors encoding forebrain (PAX6 and OTX2), midbrain (PAX6, OTX2, and EN1/PAX2), and hindbrain (EN1/PAX2, GBX2, and FGF8). The forebrain-midbrain barrier is defined by the mutually exclusive expression of PAX6 (forebrain) and EN1/PAX2 (midbrain), while the midbrain-hindbrain boundary by OTX2 (midbrain) and GBX2 (hindbrain). OTX2 and GBX2 are regulated by FGF8 expression. (c) Dorsoventral patterning with dorsal gradients for BMP4 and WNT and with a ventral gradient of SHH (Sonic hedgehog). (d) Transverse section through the neural tube depicting various neurons specified by the gradient of SHH from the floor plate and the BMP4 and WNT from the roof plate: V0–3: interneurons and MN: motor neurons.

neural induction and in repositioning morphogen gradients between the R-C and D-V axes (Figures 3(b) and 3(c)). Against this background, we and others have utilised Nodal antagonism alone to achieve neural specification from hPSCs in suspension culture [14–16, 18], although the most widely adopted approach to neural conversion from hPSCs is termed dual-Smad inhibition and utilises both Nodal and BMP4 antagonists in combination [16].

2.2. Other Factors Implicated in Neural Induction

2.2.1. Fibroblast Growth Factors (FGFs). FGFs are a diverse collection of secreted diffusible glycoproteins that act by binding with differential affinity to four classes of extracellular receptor (FGFR 1–4). The precise role of FGF signalling in neural induction remains controversial, but studies collectively suggest an early function to promote competence for neural conversion and later functions in transcriptional antagonism of BMP. Another important member of the FGF family, FGF8, is expressed in the mouse embryo in the extraembryonic ectoderm and the epiblast before and during gastrulation (Figures 3(b) and 3(c)). FGF8 activates calcineurin, which dephosphorylates Smad 1/5, the main components of the BMP4 pathway [19]. Thus, FGF8 can inhibit BMP4 signalling leading to neural induction. This finding further supports the complexity of neural induction and somewhat challenges the previous "default" model. Human PSC biology has also contributed to understanding the relevance of FGF in neural induction, with some studies demonstrating that FGF withdrawal or antagonism (together with Nodal and BMP4 antagonism) facilitates neural conversion [20–22], and others suggesting that FGF has neural inducing capacity [23–26]. These seemingly contradictory findings can be at least partially reconciled through

recognition that different culture conditions were employed in each of these studies (e.g., monolayer versus suspension culture; different programmes of coadministered extrinsic signals), which may alter the influence of FGF on neural induction in a context-dependent fashion.

2.2.2. WNT Signalling. WNTs are secreted glycoproteins responsible for establishment of the dorsoventral axis of the embryo, a direct consequence of which is the acquisition of neural identity. Administration of mRNA encoding WNTs (or their effectors) into the animal hemisphere of one-cell embryos by injection generates ectopic neural tissue. WNT signalling is itself activated by BMP4 and implicated in a Nodal positive feedback loop [27] (Figure 3(a)). The AVE secretes Dickkopf, a WNT pathway antagonist contributing initially to the R-C, and later the D-V, Nodal gradient (Figures 3(b) and 3(c)). However, WNT3 activation does not impair neural induction in mouse embryos [28], mESCs [29], and hiPSCs [30]. An extra layer of complexity is added by the different ways in which WNT can act throughout development, the canonical β-catenin pathway (to promote proliferation), or the noncanonical JNK pathway (to promote neuronal differentiation) in an FGF2-dependent manner [31].

These findings collectively suggest that neuroectoderm specification is likely more complex than the "default" (BMP4 inhibition) or "organiser" (combined BMP4, WNT3, and Nodal inhibition) models might suggest. The effects of each relevant signalling pathway are temporally regulated and determined by developmental context, justifying their systematic investigation (both individually and combinatorially) in the neural conversion of hPSCs [26].

3. Neural Patterning: An Overview

Once specified, the neuroectoderm is subsequently regionalized along the R-C axis of the embryonic body (Figures 2(c) and 4(a)). Organiser regions can be divided into those that are involved in generating rostral versus caudal structures in the neuraxis [32]. More specifically, following gastrulation the head organiser tissue lies under the prechordal neural plate (anterior neurectoderm), whereas tail organiser tissue becomes notochord and somites and lies beneath the epichordal neural plate (posterior neurectoderm). Interestingly, there is evidence that during neural induction in mESCs, WNT and FGF signalling promote neuromesodermal precursors, a population of cells that gives rise to spinal cord neurons and paraxial mesoderm [29]. Signals that inhibit BMPs (e.g., Noggin) and WNTs (e.g., Dickkopf) stimulate production of the prechordal plate, insights which have again guided ontogeny recapitulating hPSC differentiation protocols [33].

The precise timing and mechanisms of neuraxial patterning remain unresolved. A popular model is that neural induction initially specifies rostral precursors, upon which caudalising signals subsequently respecify positional identity in a progressive and stereotyped manner to establish subdivisions of the posterior neuraxis. Some of the signalling pathways implicated in neural induction also appear to play key roles in early R-C and D-V patterning at later stages [10]; they establish a matrix of positional cues (Figures 4(a) and 4(c)), which in turn influence precursor cell fate specification through graded concentrations of morphogenetic signals. In broad terms, the anterior neuroectoderm generates the forebrain, and the posterior neuroectoderm gives rise to the midbrain, hindbrain, and spinal cord [32]. The D-V signalling pathways have more pertinent roles in generating neural cell-type diversity within each of the aforementioned R-C subdivisions (Figure 4(c)). It is noteworthy that other mechanisms, such as local signals between developing neurons, also contribute to the full ensemble of neuronal subtypes. Figure 4 summarizes some of the relevant concepts here, which are explained in further detail below.

3.1. Early Patterning in the R-C Axis. Evidence from animal studies suggests that spatially and functionally distinct cell populations organise development of head and trunk structures [32]. The head organiser tissue is located in the AVE and the trunk organiser in the node and anterior primitive streak (Figure 2(a)). A wealth of evidence implicates BMP antagonism in forebrain development (Figure 4(a)). Indeed, neural conversion strategies utilising BMP antagonism in hPSCs generally report forebrain precursor specification [16, 23, 34, 35].

Studies using a range of approaches have shown that AVE is necessary for normal forebrain development with Nodal signalling being critical in this process [1]. Collectively, these studies suggest that partial reduction of Nodal signalling primarily affects specification of the prechordal mesendoderm, which is necessary for antagonising caudalising signals and thus perturbs forebrain development. Therefore, Nodal signalling is necessary for proper R-C patterning of the neuroectoderm (Figure 4(a)). Smad 2 and Smad 3 are requisite intracellular effectors of Nodal signals. Previous reports implicate Smad 2/3 in neural development; in mice, for example, Smad $2^{+/-}$ and Smad $3^{-/-}$ mutant embryos exhibit a miniaturized head-like structure [36]. In zebrafish, injection of mRNAs encoding dominant-negative Smad 2/3 mutants also results in a smaller head [37]. However, the precise roles of Smad 2/3 in neural induction and neuroectodermal patterning remain incompletely understood. Against this background and consistent with these findings, we and others have demonstrated that small molecule inhibition of Smad 2/3 imposes caudal regional identity on hPSC-derived neural precursors [15, 26].

A FGF signalling gradient operates along the R-C axis to induce the expression of paralogous Hox genes in the neural tube. Hox genes located at one end of the cluster ($3'$ end) are expressed more rostrally in response to low levels of FGF; conversely genes at the opposite end ($5'$ end) are expressed caudally in response to high levels of FGF (Figure 4(a)). Different Hox genes are consequently expressed at brachial (Hox4–Hox8), thoracic (Hox8-Hox9), and lumbar (Hox10–Hox13) levels of the neural tube [38]. The mechanisms by which a Hox-based transcriptional network choreographs these processes are now being systematically resolved [39]. These graded FGF signals regulate the primary Hox gene expression pattern before further superimposed cues refine subset-specific Hox expression. Rostrally, retinoic acid (RA)

regulates Hox expression at cervical/brachial levels, in part by antagonising the FGF gradient (Figure 4(a)). More caudally, Gdf11 (also a member of the Tgf-β superfamily) plays an important role in Hox8–Hox10 gene expression at thoracic and lumbar neural tube regions [40].

3.2. Patterning in the D-V Axis. The D-V arrangement of neuraxial anatomy is closely correlated to functional organisation. This anatomical polarity is clearly evident in the spinal cord where motor neurons reside in the ventral horns and sensory neurons are positioned in dorsal root ganglia. In the rostral neuraxis, structures such as the basal ganglia (including the substantia nigra) are ventrally located, while the cerebral cortex is dorsally positioned. R-C and D-V patterning is carefully integrated in a highly stereotyped manner. Broadly, ventral regional specification requires activation of both the Nodal and Sonic hedgehog pathways with antagonism of BMP signalling. Over and beyond its role in R-C patterning, RA is required for intermediate zone specification within the D-V axis. Likewise, FGF also plays important roles in ventral domain specification. The major contributors to D-V axis formation are BMPs and WNTs dorsally, and Sonic hedgehog ventrally [41]. Distinct neuronal subtypes are generated through interaction of opposing D-V morphogenetic gradients, which form a matrix of "coordinates" that combinatorially encode discrete precursor domains in a stereotyped D-V array [2, 3]. In the neural tube, this developmental strategy underlies motor neurogenesis and ventral interneurogenesis (Figure 4(d)). Ventral neural patterning results from morphogens originating from the floor plate and the notochord. In the early 1990s, different labs cloned vertebrate homologues of the *Drosophila* gene hedgehog, which encode secreted signalling proteins. Sonic hedgehog (SHH) transpired as the ventrally secreted morphogen conferring D-V neural tube polarity (Figure 4(c)). It is now well established through a variety of gain- and loss-of-function studies in different species that SHH plays crucial and indispensible roles in specifying ventral cell types throughout the neuroectoderm [41]. SHH is first expressed in the notochord and later the floor plate, likely secondary to auto induction (Figure 4(d)). Its function is concentration-dependent and its major effector mechanism is repression of GLI3 transcription factor. Spinal motor neuron generation, for example, depends on two temporally distinct phases of SHH signalling: an early period where it ventralizes neural plate precursors and a late period where it promotes differentiation of these precursors into motor neurons, at which point there is a concentration-dependent specification of ventral precursors into motor neurons or interneurons (Figure 4(d)).

How is positional identity imposed on precursor cells? Several studies have implicated a group of factors, predominantly the homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors, as crucial regulators here. These are expressed in strictly organised arrays along the D-V axis of the neural tube. Individual proteins are designated as classes I or II by their response to SHH signalling. Class I proteins are repressed by SHH, thus defining their ventral limit of expression, while class II protein expression is induced by SHH and defines dorsal expression boundaries. Specifically in the context of spinal cord development, such cross-repressive interactions allow the establishment of five distinct ventral precursor domains, which in turn permit the specification of distinct neuronal subtypes. Gain- and loss-of-function experiments have further supported this putative mechanism across different species, where ectopic expression of HD proteins predictably changed the regional allocation of individual neuronal subtypes within the neural tube [38, 42]. A similar cross-repressive interaction between protein classes I and II also underlies the developmental "logic" of ventral spinal neurogenesis. The most ventral aspects of neural patterning (i.e., floor plate) require Nodal signalling, and FGF has also been broadly implicated in ventral patterning within the neuraxis [41].

SHH signalling does not appear to contribute to patterning in the dorsal neural tube. However, BMPs have similar and complementary roles in dorsal patterning of the neural tube and telencephalon (Figure 4(c)). These serve as the primary dorsal morphogenetic cues by establishing a high to low concentration from dorsal to ventral positions. In a similar fashion to SHH in the ventral neural tube, this BMP gradient enables distinct precursor domains to be defined, thus permitting the generation of diverse dorsal neuronal subtypes [43].

4. Directed Differentiation of hPSCs

These aforementioned developmental studies provide a conceptual framework to rationalise both neural induction strategies and bespoke programmes of morphogenetic cues for the directed differentiation of hPSCs to clinically relevant and region specific neurons (summarized in Figure 5 and Table 1).

4.1. Forebrain. "Default" neural conversion from hPSCs to forebrain neuronal subtypes has been demonstrated in a variety of systems including chemically defined suspension culture, not requiring extrinsic signals, as well as in an adherent culture method [16, 44, 45]. These studies began in 2007 with the discovery that a *selective Rho-associated kinase* (ROCK) inhibitor permits survival of dissociated hPSCs, thus allowing systematic manipulations to cell fate after dissociation [44]. A year later, the same lab again employed serum-free embryoid body-like (SFEB) culture but this time to recapitulate cell intrinsic and temporally regulated cortical laminar determination in vitro [45]. These and subsequent studies have confirmed cortical layer specific expression of different markers including Reelin in layer 1 (Cajal-Retzius neurons), TBR1 and CTIP2 in deep layers, and SATB2, BRN2, and CUX1 in superficial cortical layers [46]. Such default dorsal telencephalic differentiation strategies tend to give rise to predominantly glutamatergic, but also some GABAergic, neurons [47].

Prior to terminal differentiation, if specified dorsal telencephalic precursors are exposed to SHH and/or a WNT antagonist, they are ventralised to generate subpallial derivatives (i.e., of the lateral and medial ganglionic eminences; LGE and MGE, respectively). Upon terminal differentiation,

Table 1

Cell type	Study	Culture method	Programme of developmental cues for neural conversion and patterning	Duration (days)
Cortical precursors	Watanabe et al. 2007 [44]	Serum-free embryoid body-like (SFEB)	BMP antagonist (BMPRIA-Fc) Activin/Nodal antagonist (LeftyA) Wnt antagonist (Dkk1)	35
Cortical neurons	Eiraku et al. 2008 [45]	SFEB derivative	BMP antagonist (BMPRIA-Fc) Activin/Nodal antagonist (LeftyA) Wnt antagonist (Dkk1)	45–60
Cortical neurons	Chambers et al. 2009 [16]	Monolayer	BMP antagonist (NOGGIN) Activin/Nodal antagonist (SB431542)	19
Cortical neurons and MGE/LGE neurons	Li et al. 2009 [47]	Suspension	None for cortical (endogenous Wnt) For MGE and LGE derivatives: Wnt antagonist (Dkk1) Sonic hedgehog (SHH)	30–35
Cortical neurons	Shi et al. 2012 [46]	Monolayer	BMP antagonist (NOGGIN) Activin/Nodal antagonist (SB431542)	80–100
Midbrain dopaminergic neurons	Kriks et al. 2011 [50]	Monolayer	BMP antagonist (NOGGIN or LDN) Activin/Nodal antagonist (SB431542) Sonic hedgehog (SHH and purmorphamine), Fibroblast Growth Factor 8b (FGF8b), Wnt agonist (CHIR99021)	80
Midbrain dopaminergic neurons	Kirkeby et al. 2012 [48]	Embryoid body	BMP antagonist (NOGGIN) Activin/Nodal antagonist (SB431542) Wnt agonist (CT99021) Sonic hedgehog (SHH-C24II)	35
Midbrain dopaminergic neurons	Jaeger et al. 2011 [52]	Monolayer	BMP antagonist (NOGGIN) Activin/Nodal antagonist (SB431542) FGF/ERK antagonist (PD0325901) Fibroblast Growth Factor 8b (FGF8b), Sonic hedgehog (SHH)	30–35
Cerebellar neurons	Erceg et al. 2012 [59]	Embryoid body	Fibroblast Growth Factors (FGF8, FGF4, and FGF2) Retinoic acid (RA) Wnt agonists (Wnt1, Wnt3a) BMPs (BMP4, BMP6, BMP7, and GDF7) Sonic hedgehog (SHH)	35
Cerebellar neurons	Muguruma et al. 2015 [57]	SFEBq	Activin/Nodal antagonist (SB431542) Fibroblast Growth Factors (FGF2, FGF19) Insulin Stromal cell-derived factor 1 (SDF-1) (co-culture with mouse granule cells to generate Purkinje cells)	35–135
Cerebellar neurons	Wang et al. 2015 [58]	Embryoid body	Fibroblast growth factor (FGF2) Insulin Sonic hedgehog antagonist (cyclopamine) (coculture with rat organotypic cerebellar slice to generate Purkinje cells)	20–65
Spinal cord motor neurons	Li et al. 2005 [63]	Monolayer	Retinoic acid (RA) Sonic hedgehog (SHH) Fibroblast Growth Factor (FGF2)	21–35
Spinal cord motor neurons	Patani et al. 2011 [18]	Suspension	Activin/Nodal antagonist (SB431542) Sonic hedgehog (purmorphamine) Fibroblast Growth Factor (FGF2)	21–35
Spinal cord motor neurons	Calder et al. 2015 [67]	Monolayer	Activin/Nodal antagonist (SB431542) BMP antagonist (LDN193189) Retinoic acid (RA)	35–40

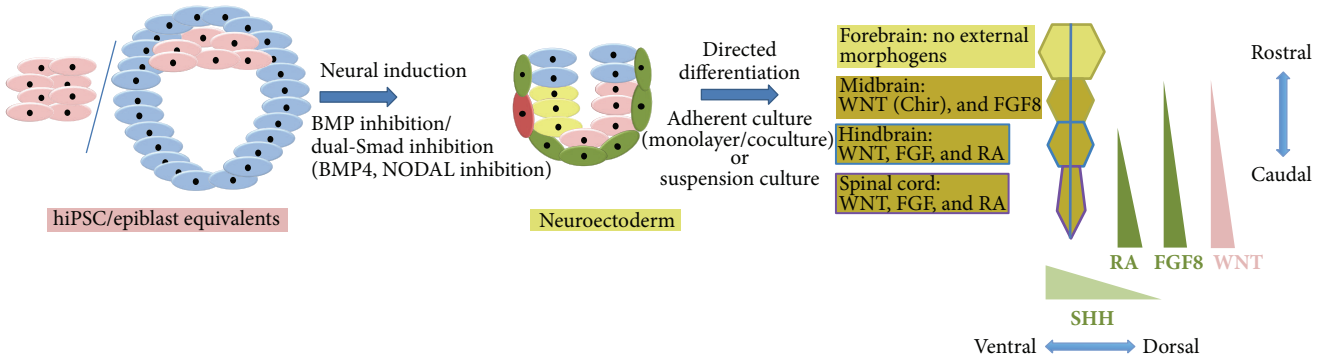

Figure 5: Methods for directed differentiation: hPSCs can be directed to undergo neural conversion by applying developmental principles of inhibiting BMP4 and/or Nodal. From neuroectoderm, differentiation of different neuraxial regions can be achieved by recapitulating developmental morphogenetic instruction: forebrain (default), midbrain (WNT, FGF8 activation), hindbrain (WNT, FGF8, and others, RA), and spinal cord (WNT, FGF8, and others, RA). These gradients are shown on the right of the figure for the rostrocaudal axis. Another important morphogenetic cue used in directed differentiation is SHH for its ventralising effect within the D-V axis. All timelines are given for mouse and human embryonic development.

these ventralised telencephalic cultures give rise to GABAergic projection neurons and interneurons. Clinically relevant cell types originate from the LGE (e.g., medium spiny projection GABAergic neurons, which are relevant to Huntington's disease and dystonia) and the MGE (e.g., basal forebrain cholinergic neurons relevant to Alzheimer's disease). Further sophistication can be added to the aforementioned directed differentiation strategies by carefully regulating SHH and WNT pathways (which orchestrate dorsoventral positional identity in this context). For example, a low concentration of SHH alone permits the specification of both LGE and MGE derivatives, whereas if a WNT antagonist is added to SHH, the more ventral MGE (i.e., NKX2.1 expressing) neurons are preferentially specified at the expense of LGE (i.e., GSX2, DLX, MEIS2, and ISLET1 expressing) neurons. Some elegant and ontogeny recapitulating strategies have been defined for the generation of authentic DARPP32 expressing medium spiny projection neurons [33, 47].

4.2. Midbrain. Differentiating hPSCs into midbrain dopaminergic neurons has maintained great enthusiasm likely owing to their potential to understand and treat Parkinson's disease. Although dopaminergic neurons exist throughout the nervous system, there is a region-specific functional heterogeneity that has been experimentally demonstrated by performing anisotopic implantation experiments [48]. Midbrain dopaminergic neurons are developmentally partitioned to three distinct nuclei: (i) the substantia nigra pars compacta (A9 group), which is primarily affected in Parkinson's disease, (ii) the ventral tegmental area (A10 group), and (iii) the retrorubral field (A8 group). Noting that hPSC-derived neural precursors have a default rostral (forebrain) and dorsal (cortical) identity, morphogen-guided positional respecification, or patterning, to the ventral mesencephalon is necessary for the differentiation of authentic midbrain dopaminergic neurons. Feeder-free and feeder-dependent differentiation approaches have both been employed to generate midbrain dopaminergic neurons from hPSCs. Feeder-dependent differentiation strategies have utilised mouse stromal cell lines (e.g., PA6), which, even though relatively easy to establish, carry the main disadvantage of being chemically undefined and animal-derived. From developmental in vivo studies, we are guided by the insight that FGF8 signalling leads to a cross-repressive interaction between Otx2 and Gbx2, defining the midbrain-hindbrain boundary (MHB; Figure 4(b)) and imparting rostrocaudal positional identity to precursors of the MHB [49]. Otx2 and Gbx2 control patterning in this region by regulating the expression of two morphogenetic cues, WNT1 in midbrain and FGF8 in the hindbrain. Furthermore, in combination with Otx2 expression, cross-repressive mechanisms between Pax6 and En1/Pax2 define boundaries of regional fate allocation to either forebrain or midbrain (Figure 4(b)).

Against this background, initial approaches to midbrain differentiation were based on FGF8 for R-C patterning to the region of the midbrain, and SHH for ventralization into dopaminergic neurons, although the yields were low (approx. 30%) using such strategies. Furthermore, subsequent studies have raised the possibility that PA6 and SHH/FGF8-based approaches alone are not sufficient to generate authentic midbrain dopaminergic neurons [50, 51]. The field then underwent a period of reevaluation where protocols that recapitulated ventral mesencephalic development with more fidelity and precision were developed. During this time, earlier protocols were systematically refined and superseded by studies using WNT agonists [48, 50], most notably from the Studer Lab who established an efficient midbrain floor plate differentiation strategy through which dopaminergic neurons were efficiently specified. Crucially, this study demonstrated functional engraftment and recovery in mice, rats, and nonhuman primates with Parkinson's disease [50]. Contemporaneous studies showed that by using canonical WNT agonists at different concentrations and for defined durations, the generation of diverse regionally specified progenitors from fore- to hindbrain is possible. Interestingly, the generated midbrain dopaminergic neurons, but not

their telencephalic counterparts, could reverse structural and functional deficits in animal models of Parkinson's disease. This subtype specificity highlights the unparalleled potential of *directed* differentiation of hPSCs in regenerative medicine [48]. A further notable study in this arena used transient blockade of FGF signalling to refine midbrain positional identity and yield authentic dopaminergic neurons with high efficiency [52]. Although it can be argued that these later studies yield more authentic midbrain dopaminergic neurons because they utilised developmentally rationalised cues, it should be noted that the GSK3β inhibitors such as CHIR99021 used here for WNT pathway activation do have off target effects (i.e., they regulate pathways other than WNT) [53]. Additionally it is noteworthy that more specific WNT pathway activators (e.g., WNT3a) do not reproducibly generate midbrain dopaminergic neurons with the same efficiency as the GSK3β inhibitor CHIR99021 [50, 54]. In future studies, the absolute requirement for GSK3β inhibition and the identification of additional key regulatory pathways would be of great importance to establish.

4.3. Hindbrain and Cerebellum. Broadly, evolutionary pathways appear to be more conserved in caudal (primitive) regions of the CNS such as the hindbrain. The hindbrain can be divided into rostral and caudal portions, which are separated by rhombomere 4 (r4). Neurons derived from rostral regions project to and innervate myriad brain regions, whereas the caudal portion, located in the myelencephalon, gives rise mainly to descending spinal projections. The brain innervating central serotonergic neurons, originating from r2-3 of the rostral raphe, contribute to higher order brain functions and are implicated in a range of psychiatric disorders. By using EGF and FGF2 in the maintenance media, so-called "long-term self-renewing rosette-type" hPSC-derived neural precursors can be expanded which exhibit a ventral anterior hindbrain-like expression profile after prolonged culture [55]. These precursors preferentially generated GABAergic neurons, some of which were serotonergic neurons. This finding likely reflects positional respecification of the default forebrain identity secondary to protracted culture in FGF2, which is known to have caudalising properties. Very recently, a protocol for directed differentiation of hPSCs to functionally validated hindbrain serotonergic neurons through activation of the WNT and SHH pathways was reported [56].

There are few reports of cerebellar differentiation with demonstration of electrophysiologically mature and functional Purkinje- and granule-cell specification [57, 58]. A recent study generated MATH1-positive cerebellar-like granule cells from iPSCs using a complex programme of sequentially administered morphogens, including FGF8, RA, FGF4, FGF2, WNT1a, WNT3a, BMP4, GDF7, BMP7, BMP6, SHH, BDNF, Jagged1, and NT3 [59]. More recently an ontogeny recapitulating strategy for cerebellar neurogenesis achieved efficient directed differentiation of hPSCs using three morphogens only [57]. Here, hPSC-derived embryoid bodies were first positionally specified to the midbrain-hindbrain boundary and subsequently directed to cerebellar plate neuroepithelium (CPNE). CPNE in turn gave rise to functionally mature Purkinje- and granule cells, DCN-neurons, and various interneurons in specific coculture settings by sequentially administering FGF2, FGF19, and SDF1. A contemporaneous study used insulin, FGF2, and an antagonist of SHH signalling (cyclopamine), again necessitating coculture with rat cerebellar slices to reinforce the validity of this approach for directed differentiation to cerebellar neurons [58]. Both of these recent studies relied to some degree on coculture with isotopic organotypic slices/rodent cerebellar derivatives. Future studies in this area should focus on overcoming reliance on coculture with rodent or human cerebellar slice cultures by identifying the requisite extrinsic signals for specifying cerebellar derivatives at each stage of their lineage restriction.

4.4. Spinal Cord. The generation of functional spinal cord derivatives, including motor neurons, has been achieved from hPSCs through a variety of approaches using insights from developmental biology [15, 60–62]. These strategies employed either simultaneous or sequential administration of caudalising (e.g., RA) and ventralising (e.g., SHH) morphogens prior to terminal differentiation. These studies confirmed the expression of specific motor neuron fate determining factors including HB9, specific enzymes/transporters including choline acetyltransferase (ChAT) and the vesicular acetylcholine neurotransmitter transporter (vAChT), and also coculture with myotubes to demonstrate the formation of physiologically relevant neuromuscular junctions [18, 60, 63]. Electrophysiological studies confirm that hPSC-derived motor neurons acquire appropriate functional properties [60]. Motor neuron precursors have importantly been shown to survive and integrate in rodent embryonic spinal cord [64, 65] and to project axons forming physiological synapses.

Treating cultures with RA typically results in a cervical or brachial positional identity [18, 65]. More caudal (lumbar) motor neuron fates can also be achieved in the absence of RA signalling, likely in response to FGF2; indeed we have reported a retinoid independent strategy for motor neurogenesis from hPSCs that yields a lumbar spinal subtype identity and favours medial motor columnar specification [18]. This retinoid-mediated diversification of motor neuron subtypes was further supported by a parallel study using mouse embryonic stem cells [65]. A recent study employed combined retinoic acid and WNT agonism to generate cranial motor neurons from hPSCs [66]. Yet another subsequent study reported the derivation of motor neurons under RA treated but SHH free conditions, uncovering important insights into human motor neurodevelopmental biology [67].

4.5. Neural Crest. Neural crest cells are highly migratory and give rise to myriad differentiated cell types including (i) sensory and autonomic neurons and Schwann cells, (ii) chromaffin cells in the adrenal medulla, (iii) melanocytes, and (iv) cranial skeletal and connective tissue components. The fate of the neural crest cells is largely determined by where they migrate to/settle. From an hPSC perspective, striking phenotypic consequences have been demonstrated based on plating density, and this provides a strategy to

generate neural crest derivatives. A high plating density favours PAX6 expressing central nervous system precursors while low plating density specified neural crest-like differentiation [16]. Using variations of this approach, stage-specific isolation/differentiation of hPSC-derived neural crest cells has been achieved using a combination of in vitro expansion, directed differentiation via extrinsic signals and cell sorting. For example, serum-free conditions with subsequent bespoke programmes of extrinsic cues can permit specification of Schwann cells, autonomic or sensory neurons, while serum based approaches tend to favour mesenchymal derivatives including adipocytes, osteocytes, chondrocytes, and smooth muscle. Functional validation has been demonstrated by transplantation of hPSC-derived neural crest cells into a chick embryo, where they exhibit preserved neural crest identity in the context of survival, migration, and differentiation [68].

5. Concluding Remarks

The unrivalled complexity of the mammalian central nervous system is enabled by a series of progressive and sequential events during embryogenesis. The degree of interconnectedness within the central neuraxis is somewhat surprising given its impressively precise organisation into discrete regions. Evolutionary conservation of developmental processes underlying the organisation of such discrete neural regions becomes increasingly less applicable to more rostral (i.e., evolutionarily "newer") components, like the forebrain. The hPSC platform is emerging as an important reductionist in vitro system to interrogate aspects of human development, which have remained experimentally inaccessible until now.

Current approaches towards such directed differentiation of hPSCs often fail to capture the dynamic and overlapping nature of neurodevelopmental processes. For instance, neural induction and patterning are often conceptualised as mechanistically distinct processes. However, a bias towards different regional fates will likely be determined by the neural conversion paradigm employed. Similarly current differentiation strategies do not yet fully acknowledge or exploit the ability to influence cell (subtype) fate decisions *postmitotically*, which has been reported [69–71]. As such, the field's approach to directed differentiation to individual cellular subtypes could potentially benefit from being more closely aligned to each respective stage of neurodevelopment, leading to bespoke conditions for each stage of lineage restriction (i.e., neural conversion, patterning, and terminal differentiation).

Developmental principles are a crucial resource for defining ontogeny recapitulating directed differentiation protocols for hPSCs (Figure 5). In addition to the wealth of knowledge that already exists from rodent developmental biology, there is an increasing number of publicly available human brain region-specific and transcriptome-wide datasets from studies using a diverse range of tissue from fetal through to adult stages [72–74]. In addition to highlighting the maturational status of hPSC-derived neurons [75], such developmental/stage-specific data sets could now serve as a gold standard for validating directed differentiation protocols to region-specific cell types. Indeed these datasets should eventually contribute to experimental design when a relatively unexplored region of the nervous system is being investigated using hPSCs. The utilisation of human brain-derived data bypasses potential issues of evolutionary divergence between mouse and man, especially in the more rostral (evolutionarily newer) regions of the neuraxis. Coupling insights gained from these invaluable resources together with high throughput platforms for protocol discovery would be a future avenue for improving the robustness of current directed differentiation strategies [66].

Finally, the hPSC field stands to benefit from defining multiple directed differentiation protocols that employ closely aligned methods for neural conversion and similar protocol durations. This may then permit more meaningful comparison between region-specific neurons, without the potentially confounding issue of differential cellular maturation. Indeed such an approach was recently utilised to show region-specific phenotypes using iPSCs derived from patients with Alzheimer's disease and motor neuron disease [76]. Taken together, such standardizations in directed differentiation of hPSCs may help to drive the identification of robust strategies to specify enriched populations of all clinically relevant region-specific subpopulations of human neurons for further study.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Alexandra Zirra and Sarah Wiethoff performed literature research, contributed to writing of the paper, and constructed figures. Rickie Patani directed the analysis and wrote the paper.

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