Using organoids to study human brain development and evolution

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Abstract
Recent advances in methods for making cerebral organoids have opened a window of opportunity to directly study human brain development and disease, countering limitations inherent in non-human-based approaches. Whether freely patterned, guided into a region-specific fate or fused into assembloids, organoids have successfully recapitulated key features of in vivo neurodevelopment, allowing its examination from early to late stages. Although organoids have enormous potential, their effective use relies on understanding the extent of their limitations in accurately reproducing specific processes and components in the developing human brain. Here we review the potential of cerebral organoids to model and study human brain development and evolution and discuss the progress and current challenges in their use for reproducing specific human neurodevelopmental processes.

KEYWORDS
Cerebral organoids, human cortical development, human cortical evolution, human embryonic neurodevelopment

1 | THE NEED FOR A HUMAN-CENTRIC APPROACH

Much of our knowledge about human brain development is extrapolated from studies of brain development in other species. It is undoubtedly true that many of these extrapolations are likely to be valid, given the high level of conservation of brain structure and function among mammalian species, but the approach has clear limitations. Most obvious is the high level of sophistication of the mature human brain compared with that of even our closest living relatives. During embryogenesis, important anatomical differences emerge between the developing brains of humans and other mammals such as the neocortex expansion and the arising of gyrification seen in higher evolved species (Florio & Huttner, 2014; Lui et al., 2011). Although many of the mechanisms that create the human brain are highly conserved, the generation of its unique attributes are likely to involve important genomic regions that show differences unique to humans, e.g., human accelerated regions (HARs), or human-specific genomic rearrangements (Bae et al., 2015; Dennis & Eichler, 2016; Gittelmann et al., 2015; Hubisz & Pollard, 2014; O’Bleness et al., 2012; Pollard, Salama, King, et al., 2006; Pollard, Salama, Lambert, et al., 2006; Prabhakar et al., 2006; Silver, 2016; Zhang & Long, 2014). These human-specific genomic features are almost certainly involved in human-specific gene regulatory
mechanisms that account for the morphological, physiological, and behavioral divergence between our brains and those of our closest relatives (Britten & Davidson, 1969; King & Wilson, 1975). These considerations lead to the inevitable conclusion that gaining a full understanding of the mechanisms regulating human brain development will require studies of human brain development.

The discovery that brain-like structures, cerebral organoids, can be made by differentiating stem cells that offers the potential to study aspects of human brain development directly. Cerebral organoids are 3D cell aggregates derived from pluripotent stem cells (PSCs) which contain many of the cell types found in embryonic brains, locally organized and behaving similarly to cells found in vivo (Eiraku et al., 2008; Kadoshima et al., 2013; Lancaster et al., 2013). Their use has already led to the identification of human-specific features of brain development, such as human-specific gene expression along cortical lineages (Kanton et al., 2019; Pollard et al., 2006). Further examples will be discussed at the end of the review. Here we review the potential of cerebral organoids to model the development of the human brain, to explore its mechanisms of development and their evolution.

## 2 | STRATEGIES FOR MAKING CEREBRAL ORGANOIDS

Brain development is a complex process involving intricate spatiotemporal regional patterning, precisely choreographed cell migration and accurately directed axonal targeting. These processes result in the production of highly specialized brain regions, each containing a specific set of cells of diverse classes with distinct functions interacting via complex circuitry. Clearly, there is no expectation that organoids will deliver a model that faithfully reproduces all aspects of brain development in their entirety. The hope is that cerebral organoids will offer models reproducing specific processes occurring in, and specific components of, the developing human brain with sufficient accuracy to make them useful for studying human brain development and disease (Le Bail et al., 2021; Shi et al., 2021).

Protocols for generating human cerebral organoids fall into two main categories. In the first, PSCs are aggregated and allowed to free-pattern and differentiate in the absence of specific added differentiation cues. Such protocols, exemplified by Lancaster et al. (2013), produce organoids comprising multiple regions corresponding to various components of the brain, such as dorsal and ventral forebrain, hindbrain, hippocampus or choroid plexus (Lancaster et al., 2013). Alternatively, many protocols include the addition of specific cues that promote formation of a specific region of the brain (Birey et al., 2017; Giandomenico et al., 2019; Paçca et al., 2015; Qian et al., 2016; Xiang et al., 2017, 2019). In a hybrid approach, multiple organoids each representing a different specific brain region are combined to generate “assembloids”, thereby achieving selective regional heterogeneity in a relatively controlled manner (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017, 2019). Generation of localized signaling centers has also been used to direct organoid differentiation (Cederquist et al., 2019; Takata et al., 2017). We discuss some of the progress and current challenges in using cerebral organoids to model components of the developing human brain, to examine particular processes involved in human brain development, and to study human brain evolution.

## 3 | USING ORGANOIDS TO PROBE EARLY HUMAN NEURODEVELOPMENTAL PROCESSES

The generation of organoids whose heterogeneous cellular composition reflects that of human fetuses has allowed us to probe many stages of human brain development, some of which predate the emergence of the brain as a discrete structure.

### 3.1 | Gastrulation and neural induction

Early in development the embryo undergoes gastrulation, during which it reorganizes to form the endoderm, mesoderm, and ectoderm, after which neural induction occurs to generate the neuroectoderm. In humans, gastrulation occurs around the end of the second embryonic week. At this stage, human embryos are difficult to obtain and cannot be cultured, for technical, legal, and ethical reasons. In many stem cell in vitro systems, neural induction can be mimicked by blocking the TGFβ and BMP signaling pathway using dual SMAD inhibition (Chambers et al., 2009). Recently, human “gastruloids” with the ability to form the three germ layers and displaying patterned gene expression have been grown in culture for the first time (Moris et al., 2020). Gastruloids generate derivatives of the three germ layers and express markers of early neuroectoderm in the absence of SMAD inhibition. Gastruloids successfully elongate and establish and maintain axial patterning, with BMP pathway-related genes expressed preferentially anteriorly and WNT pathway genes expressed preferentially posteriorly (Moris et al., 2020). Due to technical limitations, gastruloids have only been cultured for up to 3 days, providing insufficient time for the neuroectoderm to generate cell types found in the developing brain. Nevertheless, they offer a powerful new three-dimensional
model of very early human developmental processes, predating the start of brain development, that have previously been difficult or impossible to study.

3.2  |  Rostral-caudal regionalization

In vertebrates, gastrulation and generation of neuroectoderm is followed by folding and closure of the neural plate to form the neural tube. Regional specialization of the neural tube along its rostral-caudal (R-C) axis determines the prospective brain and other CNS regions. The mechanism by which R-C regionalization occurs in humans remains a mystery. In non-human species, one major signaling pathway implicated in R-C regionalization is Wnt signaling, with many Wnt signaling genes showing spatially restricted expression patterns during early brain development (Fischer et al., 2007; Hoang et al., 1998; Roelink & Nusse, 1991; Rowitch & McMahon, 1995). The addition of Wnt agonists caudalizes neuroectodermal cells, including those derived from human stem cells, in a dose-dependent manner (Fang et al., 2019; Imai et al., 2015; Kiecker & Niehrs, 2001; Kirkeby et al., 2012; Nordström et al., 2002; Rhinn et al., 2005; Rifes et al., 2020). Notably, in the experiments of Rifes et al. (2020) on human cells, expression of regionalized markers appeared very early, after only a day of differentiation, a day before neural-specific genes became expressed, suggesting that regionalization occurred before neural specification. This interesting result needs further investigation to establish whether it is a consistent finding.

The importance of WNT signaling in the R-C regionalization of the human neuroectoderm remains unclear. In non-human species, the use of 3D culture systems in which aggregates of stem cells self-organize along an axis has made it possible to investigate these processes. Studies using aggregates of mouse embryonic stem cells (mESCs) revealed that after only 3 days of culture, aggregates showed localized Wnt activity (ten Berge et al., 2008) and self-organized along the R-C axis (Takata et al., 2017). This was preceded by expression of Fgf5, inhibition of which prevented polarization and produced rostralized aggregates. Activation of Fgf led to caudalization and increased expression of Wnt genes. This work revealed that an interplay between Fgf and Wnt signaling in neuroectoderm is key to R-C patterning, suggesting that the initial appearance of a localized population of Fgf5+ cells induces and combines with localized expression of Wnt signaling components to repress rostral fates caudally. In the human gastruloid study (Moris et al., 2020), pre-treatment of hPSCs with the WNT activator CHIR99021 was essential for axis formation and could not be replaced with WNT3A and BMP4. Although it is possible to generate a R-C axis in vitro models of human neural differentiation, these models all require extrinsic signals (Cederquist et al., 2019; Rifes et al., 2020). Further insights from these models may provide answers to the exact cellular signals required for axis formation during early human brain development. Furthermore, being able to recapitulate embryo-like axes within organoids may produce a better model of normal brain structure.

3.3  |  Dorsal-ventral regionalization

It is suspected that endogenous forebrain organizing centers that direct dorsal-ventral (D-V) patterning arise spontaneously in some free-patterning forebrain human organoids (Lancaster et al., 2013; Renner et al., 2017). The evidence in favor of this possibility is the observation in such organoids of localized expression of signaling molecules important for D-V patterning such as WNT2B and BMP6 (Renner et al., 2017). Moreover, the presence of such signaling centers has been inferred from the fact that regions mimicking those specified along the D-V axis of the embryo are generated in these organoids. For example, regions with molecular identities typical of ventral telencephalic regions, including lateral, medial, and caudal ganglionic eminences (LGE, MGE and CGE), or regions corresponding to components of dorsal telencephalon, such as choroid plexus or hippocampus, have been identified (Hansen et al., 2013; Kim et al., 2019; Lancaster et al., 2013; Ma et al., 2013; Renner et al., 2017; Slope et al., 2018). Even though free-patterning organoids can develop organizing centers and regions with the characteristics of those that develop at different locations along the D-V axis, current models have not yet succeeded in reproducing accurately the spatial layout of those centers and regions in the developing embryo. Achieving this will most likely require the application of external cues in a spatiotemporally controlled way.

4  |  USING ORGANOID TO PROBE LATER HUMAN NEURODEVELOPMENTAL EVENTS

4.1  |  Cerebral cortical development

The cerebral cortex forms in the dorsal part of the forebrain. Cerebral cortex-like tissue is a consistently generated component of free-patterning organoids and stem cell-derived neuronal models, suggesting that its production is a default state achieved in the absence of externally applied molecular cues (Table 1) (Gaspard et al., 2008; Smukler et al., 2006). A large amount of research using human cerebral organoids has focused on the analysis of cerebral cortical development.

In the developing human cortex, the generation of neurons from progenitors known as radial glia follows a highly conserved intrinsically regulated differentiation program...
| Region          | Externally applied patterning molecules               | General early markers                                                                 | Neuronal markers                                                                 | Notable features                                                                 | References                                      |
|-----------------|------------------------------------------------------|----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------|------------------------------------------------|
| Pallium         | PAX6, OTX2, FOXG1 (Forebrain); Emx1 (Dorsal cortex); PAX6, SOX2 (RGs/VZ); FAM107A (oRGs); TBR2 (IPs) | Tuj1 (new-born); NeuN (differentiating); CTIP2, TBR1 (early born, deep-layers); SATB2 (late born, upper-layers); CUX1, BRN2 (layer II/III); REELIN (Cajal-Retzius cells) | Display well-defined laminar neuronal zones: VZ, SVZ and CP                     | Generate diverse electrophysiologically active neuronal subtypes of all six cortical layers | Krefft et al. (2018); Lancaster et al. (2017); Pașca et al. (2015); Qian et al. (2016); Renner et al. (2017); Xiang et al. (2017); Yoon et al. (2019) |
| Sub-pallium     | WNT inhibition, enhanced SHH signaling                | GSX2 (dLGE, CGE); NKKX2-1 (MGE); LHX6 (subregion of MGE)                           | GABA, GAD67 (GABAergic markers); SST, CR, CB, PV (IN subtypes); SYN1, VGAT (synaptogenesis) | Develop ventral forebrain GE subregions that produce different interneuron subtypes with an ongoing process of synaptogenesis Generate functional neurons: ~75% of neurons generated action potentials in response to depolarization and ~60% exhibited spontaneous inhibitory postsynaptic currents (IPSCs) that reverse in direction around the chloride reversal potential and are abolished by gabazine, a GABAA receptor antagonist | Bagley et al. (2017); Biey et al. (2017); Sloan et al. (2018); Xiang et al. (2017) |
| Hippocampus     | manipulation of Wnt and bone morphogenetic protein (BMP) signaling | Nrp2, Lef1 (NE layers); Zbtb20 (hippocampal neurons and their precursors)          | Prox1 (granule neurons); KA1 (CA3 pyramidal neurons); CaMKII (mature hippocampal neurons) | Neurons capable of forming neuronal networks with functional synaptic connections: voltage-dependent Na–K currents, action potential following injection of depolarizing currents, spontaneous excitatory postsynaptic currents present | Sakaguchi et al. (2015)                         |
| Region          | Externally applied patterning molecules | General early markers | Neuronal markers                                                                 | Notable features                                                                 | References                                      |
|-----------------|----------------------------------------|-----------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------|------------------------------------------------|
| Thalamus        | BMP7 and MEK inhibitors, dual SMAD inhibition | OTX2, TCF7L2, and GBX2 (thalamic identity) |                                                                                  | Reciprocal thalamocortical (TC) connections observed when fused with cortical organoids; Functionally active neurons with synchronized calcium surges without stimuli | Shiraishi et al. (2017); Xiang et al. (2019) |
| Midbrain        | SHH agonists, FGF-8, dual-SMAD inhibition, GSK3b inhibitor | FOXA2, NESTIN (floor plate progenitor), MASH1, OTX2 (midbrain progenitors), NURR1 (IZ postmitotic cells) | MAP2 (mantle zone neurons); TH, FOXA2, DAT, NURR1, PITX3 (dopaminergic neurons); TH, GIRK2, CB (A9 and A10 midbrain dopaminergic neurons) | Generate functional neurons: fire rhythmically, respond to treatment with dopamine D2/D3 receptor agonists, form intricate neural networks that can be probed by electrical stimulation | Jo et al. (2016); Qian et al. (2018) |
| Cerebellum      | FGF2, insulin, FGF19, SDF1 | EN2, GBX2 (midbrain-hindbrain markers); FGF8, WNT1 (isthmic organizer); PTF1A, KIRREL2 (cerebellar plate neuroepithelium) | L7, SKOR2, OLIG2, LHX5 (Purkinje cells); NRGN, PAX2, GAD65 (Golgi cells); ATOH1, BARHL1, PAX6 (Granule cells); LHX2, TBR1, SMI32 (DCN); PV, CB, GAD65 (INs) | Generate mature Purkinje cells after long-term culture that exhibit functional electrophysiological properties: spontaneous repetitive firing, Is currents and AMPA-receptor-dominant response to glutamate input | Muguruma et al. (2010, 2015) |

Abbreviations: CGE, caudal ganglionic eminence; CP, cortical plate; DCN, deep cerebellar nuclei; dLGE, dorsal lateral ganglionic eminence; IN, interneuron; IPs, intermediate progenitors; IZ, intermediate zone; MGE, medial ganglionic eminence; MZ, marginal zone; NE, neuroepithelium; RGs, radial glia, SP, subplate, SVZ, subventricular, VZ, ventricular zone.
(Figure 1) (Götz & Huttner, 2005; Mayer et al., 2018; Nowakowski et al., 2017), with interneurons (appearing in the subpallium before migrating into the cerebral cortex) and maturing later than excitatory neurons (around gestational weeks 26 and 16 respectively) (Zhong et al., 2018). Organoid protocols can generate the major cell types identified in the developing human cortex between gestational weeks 6 and 26, including neuroepithelial cells, radial glia, outer radial glial cells (oRGs), intermediate progenitors, immature neurons, cortical excitatory and inhibitory neurons, astrocytes, and oligodendrocytes (Figure 1) (Bhaduri et al., 2020; Fiddes et al., 2018; Giandomenico et al., 2019; Quadrato et al., 2017; Tanaka et al., 2020; Velasco et al., 2019; Xiang et al., 2017; Zhong et al., 2018). Organoids also recapitulate known cellular features of in vivo neurogenesis such as interkinetic nuclear migration and radial migration (Figure 1) (Bershteyn et al., 2017; Qian et al., 2018, 2020). They present a valuable opportunity to investigate processes, such as the cellular behavior of the oRG population, that are essential for human cortical expansion and might be affected in neurodevelopmental disorders (Bershteyn et al., 2017).

Although these studies found broad correspondence between events unfolding in cortical organoids and embryonic cortex, future work will need to address the challenge raised by recent work suggesting that, at a more detailed level, the molecular identities of layer-specific and other differentiating cell populations in cortical organoids do not recapitulate the molecular signatures of the equivalent cell populations in vivo (Bhaduri et al., 2020). Furthermore, these authors found that cortical layers in organoids only display a rudimentary spatial separation, suggesting that neuronal migration is not optimized. Existing protocols appear to generate cortical organoids with important limitations for the study of later events in corticogenesis.

4.2 | The development of other brain regions

Unlike the free-patterning methods of generating cerebral organoids, adding growth factors or morphogens allows for their directed differentiation into specific brain regions (Figure 2). Table 1 summarizes the patterning molecules used to generate region-specific human organoids, the markers used to recognize them and their features.

4.2.1 | Subpallium

A subpallial identity is promoted in organoids subject to a combination of WNT inhibition together with enhanced SHH signaling. This identity is evidenced by gene expression profiles characteristic of the ganglionic eminence (GE) subregions that produce different interneuron subtypes (Bagley et al., 2017). Cytoarchitectural examination of subpallial
organoids revealed SOX2+ radial glial-like progenitors arranged in a layer resembling the embryonic ventricular zone (VZ) surrounded by an outer subventricular zone (OSVZ) and, at early stages, an inner subventricular zone (ISVZ)-like layer. The thickness of the VZ-like layer decreased during organoid development, mimicking a similar decrease during in vivo subpallial development (Birey et al., 2017; Hansen et al., 2013; Xiang et al., 2017). The subpallial organoids generated inhibitory interneuronal subtypes with functional activity in temporal patterns similar to those observed in vivo, including somatostatin (SST), calretinin (CR), calbindin (CB), and parvalbumin (PV) interneurons (Birey et al., 2017; Sloan et al., 2018; Xiang et al., 2017). The fusion of subpallial organoids with cortical organoids to generate so-called assembloids has allowed the study, with methods including time-lapse imaging, of the migration of inhibitory GABAergic interneurons from the subpallium into the cerebral cortex (Bagley et al., 2017; Birey et al., 2017; Sloan et al., 2018; Xiang et al., 2017).

4.2.2 | Striatum

Using a combination of Activin A, agonist of retinoid X receptors and inhibition of the WNT pathway using IWP-2 generate 3D spheroids that expressed high levels of the forebrain marker FOXG1 and LGE (which gives rise to the striatum) markers of DLX5, GSX2, CTIP2, and MEIS2. After culturing these spheroids for more than 80 days, scRNA-sequencing, morphological, and electrophysiological analyses showed that these spheroids contain medium spiny neurons that were electrophysiologically active. These striatal organoids were then co-cultured with cortical organoids to form cortico-striatal assembloids. In these assembloids, they found axonal projections from the cortical organoid which then formed synaptic connections (Miura et al., 2020).

4.2.3 | Hippocampus

The manipulation of WNT and bone morphogenetic protein (BMP) signaling can induce cerebral organoids to adopt a fate mimicking that of dorsomedial telencephalon, where the hippocampus forms (Chen et al., 1997; Sakaguchi et al., 2015; Xie et al., 2010). Upon dissociation, these organoids can generate hippocampal-like mature CA3 and granule neurons resembling those of the CA and DG, as well as astrocyte-like cells (Arnold & Trojanowski, 1996; Sakaguchi et al., 2015). The neurons in hippocampal organoids are also able to form

FIGURE 2 Generation of region-specific brain organoids to model regional interactions of the human brain. Despite the advancements in organoid protocols, cerebral organoids reaching a few millimeters in size are still far from resembling a fully functional human brain with interconnected regions. Using growth factors or morphogens to direct the differentiation of PSCs into region-specific organoids, however, has enabled us to recapitulate individual brain regions separately, which can then be fused into assembloids to study cellular interactions and connectivity between the different regions, bringing us one step closer to mimicking the spatial complexity of the human brain, albeit the limitations. It might be then worth generating assembloids from more than two different brain regions to gain further insights into how well regional organoids resemble the developing brain and expand on the uses of this in vitro model. For region-specific patterning molecules, markers, and features please see Table 1
neuronal networks with functional synaptic connections (Sakaguchi et al., 2015).

4.2.4 | Thalamus

The addition of BMP7 and MEK inhibitors generates organoids that contain neurons with thalamic identity, which are functionally active (Shiraishi et al., 2017; Xiang et al., 2019). Single-cell RNA sequencing (scRNA-seq) revealed that thalamic organoids contain cells that express high levels of OTX2, TCF7L2, and GBX2, markers of thalamic identity. However, markers of other brain regions such as cortex or medial ganglionic eminence (MGE) are also expressed. The thalamus and MGE form in relative proximity, and these findings suggest overlap in the molecular cues that induce cell fates in these regions.

4.2.5 | Midbrain

Application of a combination of SHH agonists, FGF8, SMAD inhibitors, and GSK3β inhibitor induces the differentiation of human iPSCs into organoids with characteristics of midbrain (Arenas et al., 2015; Jo et al., 2016; Marton & Pașca, 2016). At later stages, these organoids produce mature, functional tyrosine hydroxylase-expressing (TH+) dopaminergic neurons. A population of TH+cells also express additional markers that are enriched in A9 and A10 midbrain dopaminergic neurons (Jo et al., 2016; Marton & Pașca, 2016).

4.2.6 | Cerebellum

In the presence of high fibroblast growth factor 2 (FGF2), insulin, FGF19, and SDF1, cellular aggregates develop into cerebellum-like organoids displaying an elongated, polarized cerebellar neuroepithelium with a rhombic-lip region containing granule progenitors at one end and a three-layered cytoarchitecture that is reminiscent of the embryonic cerebellum in the first trimester (Muguruma et al., 2015). They develop cerebellar cell types including Golgi cells, granule cells, deep cerebellar nuclei (DCN) projection neurons, and Purkinje cells (Muguruma et al., 2015; Pasca, 2018). After long-term culture, mature GABA-releasing Purkinje cells bearing single long axons, elaborate dendritic branches and spines with functional electrophysiological properties appear, mimicking mature Purkinje cells in embryonic cerebellar plate (Morales & Hatten, 2006; Muguruma et al., 2010, 2015; Raman & Bean, 1999; Williams et al., 2002).

4.3 | Neural circuit formation

Functional neural circuits are formed through the stepwise process of cell migration, axonal projection, synapse formation, and synapse elimination (Matsui et al., 2020). Recent studies have found that several of those steps can be studied using organoids. The integration of inhibitory GABAergic interneurons into functional neural circuits with excitatory glutamatergic neurons in fused pallial-subpallial assembloids has been demonstrated (Birey et al., 2017; Xiang et al., 2017), showing that neurons in organoids are capable of cell migration, axonal projection, dendritic growth, and synapse formation. Similarly, other studies have reported reciprocal connections between thalamic and cortical organoids in thalamocortical assembloids. This work produced the hypothesis that neuronal circuits play a role in the maturation of the intrinsic properties of thalamic neurons, because it was found that fusion of thalamic organoids with cortical organoids increased the frequency of electrophysiologically mature neurons in the thalamic tissue (Xiang et al., 2019). Furthermore, other studies found that neurons in organoids show spontaneous network formation producing periodic and regular oscillatory events dependent on glutamatergic and GABAergic signaling, reaffirming that neurons in organoids develop axons, synapses, and mature functional properties (Birey et al., 2017; Izsak et al., 2020; Trujillo et al., 2019; Xiang et al., 2017, 2019). A recent study has pushed the boundaries of using organoids to model neural circuit formation even further by making cortico-motor assembloids that comprised of three organoids fused together (cortical-spinal-muscle). They showed that corticofugal neurons project and connect with spinal spheroids, whereas spinal-derived motor neurons connect with muscle cells. When they stimulated the cortical spheroids, they found that it triggers robust contraction of the muscle cells. Their work found the possibility of using organoids to model a multi-synaptic circuit (Andersen et al., 2020). However, organoids have not been used to investigate the process of synapse elimination or synaptic pruning. This is because during the process of neural circuit formation, inactive synapses are removed mainly by microglial cells (Paolicelli et al., 2011; Schafer et al., 2012), and brain organoids are not expected to have microglial cells as they are yolk sac-derived mesodermal cells (Ginhoux et al., 2013) whereas brain organoids are established by induction of the neuroectoderm.

5 | CURRENT CHALLENGES

Organoids have enormous potential, but their effective use depends critically on an understanding of existing limitations. Here we highlight some major current challenges.
5.1 | Cellular stress

Bhaduri et al., found that the molecular signatures of developing cortical layer-specific cell populations are not recapitulated in cortical organoids, and proposed that this was due partly to environmentally induced cellular stress. Their evidence was that cells in the organoids activated glycolysis and endoplasmic reticulum (ER) stress genes, whereas this was reversed when the organoid cells were transplanted into living brains (Bhaduri et al., 2020). Their conclusion was supported by another recent study that has analyzed transcriptomic data from most of the published cortical organoid protocols (Tanaka et al., 2020). By contrast, in vivo fetal tissue showed little expression of stress-related genes at corresponding developmental stages (Bhaduri et al., 2020; Tanaka et al., 2020).

Dysregulation of glycolysis and ER stress genes in organoids has been attributed to the depletion of nutrients and inadequate oxygen diffusion to the organoid core due to the lack of vascularization (Pollard et al., 2006; Tanaka et al., 2020). A recent study has found a possible solution to this and it involves slicing forebrain organoids during the culture period, thereby exposing the core (Qian et al., 2020). This addressed the problem of interior hypoxia and reduced cell death. The cytoarchitecture of the cortical layers was much improved, with clear separation of layers and improved cellular subtype marker gene expression. Furthermore, they found morphologically distinct astrocyte subtypes in laminar distributions resembling those in the developing human cerebral cortex (Qian et al., 2020). Another possible solution might involve the use of microfluidic perfusion systems and the addition of vascular endothelial cells, not only to overcome the limits on organoid growth potential but also to provide a means to investigate the cross-talk between neuronal and non-neuronal tissue, a feature that current models lack (Lancaster & Knoblich, 2014; Qian et al., 2016). Hybrid neurovascular spheroids have been generated and they might provide insight into such interactions (Song et al., 2019).

5.2 | Developmental bypasses

As discussed above, the broad categories of early developing cell types produced in human cortical organoids represent those found in human embryonic cortex (Bhaduri et al., 2020; Birey et al., 2017; Qian et al., 2020; Quadrato et al., 2017; Tanaka et al., 2020; Velasco et al., 2019; Xiang et al., 2019), but an important question for understanding developmental mechanisms is whether equivalent compositions are achieved through different routes. Analysis of scRNA datasets from different cortical organoid protocols alongside those from human fetal tissue suggested that multiple protocol-dependent developmental bypasses occur in the organoids (Figure 3), most likely outlining alternative differentiation routes that could exist during brain development (Tanaka et al., 2020). Three different bypasses with distinct transcriptional dynamics have been highlighted by Tanaka et al., during the differentiation trajectory into post-mitotic neurons and glia from proliferative neuroepithelial cells. Although some differentiation routes, such as those enriched for cell cycle regulators or insulin response genes, seemed to be protocol-specific, another bypass enriched for proliferation genes and early neurogenesis transcription factors was universally adopted in all protocols and considered a “major” neuronal differentiation route. Given the shorter developmental periods, the absence of cross-talk with non-neuronal tissue and the evidently smaller size and number...
of cells compared with a fully developed embryonic brain, it is possible that such alternative differentiation routes are selectively induced during organoid development to achieve this broad cell diversity, highlighting a highly constrained cell-fate transition. We do need to caution that despite the stark resemblance between region-specific brain organoids and the human brain, it remains unclear whether the in vitro developmental trajectory exactly recapitulates in vivo development and maturation processes especially during later development.

5.3 | Scalability

The need for laborious manual manipulation of organoid cultures, their heterogeneity, and batch-to-batch variation make it difficult to adapt current organoid culturing protocols for high-throughput applications (Ao et al., 2020; Lancaster et al., 2017; Marton & Pașca, 2016). To try to overcome this, some laboratories have directed their efforts toward the design of synthetic matrices that correctly mimic the human brain’s external niche. Information on the mechanical properties of brain tissues as well as the molecular composition of the extracellular matrix (ECM) has led to the synthesis of new synthetic hydrogels that can help produce organoids with less variability and higher reproducibility (Oksdath et al., 2018; Tekin et al., 2018). Microglia are critical components of the brain niche, and their absence in organoids remains a limitation that needs to be overcome (Bejoy et al., 2019; Song et al., 2019). This may be overcome by allowing them to develop within the organoid by omitting SMAD inhibition (Ormel et al., 2018) or by co-culturing organoids with iPSCs-derived microglia (Song et al., 2019).

6 | THE EVOLUTION OF NEURODEVELOPMENTAL MECHANISMS

Cerebral organoids are emerging as a powerful tool to study the differences between developing human and non-human primate (NHP) brains (Mostajo-Radji et al., 2020). Neurogenesis takes significantly longer in humans than in NHPs (Zhu et al., 2018), demonstrated by comparisons between human with NHP in both organoid and 2D culture systems, which have consistently shown that human-derived neural cells undergo more rounds of cell division, slower rates of migration and maturation, and delayed onset of neural activity (Marchetto et al., 2019; Muchnik et al., 2019; Otani et al., 2016). Cerebral organoids offer the potential to explore the mechanisms responsible for intrinsic differences such as these in the cells of humans versus those of other species. Here we illustrate this potential with some recent examples.

The ability of organoids to preserve gene regulatory networks and developmental processes allows human-specific gene expression patterns to be investigated. Pollard and et al. (2006) compared gene expression in human fetal tissue, human cerebral organoids, chimp cerebral organoids and macaque fetal tissue. They identified 261 genes whose expression levels were different in both human fetal and organoid samples when compared with both chimpanzee organoids and macaque fetal tissue; many of these genes were overlapping recent genomic segmental duplications (Pollard, et al., 2006). Interestingly, the expression of 85% of these genes was specific to cortical development. They included regulators of PI3K-AKT-mTOR signaling—a complex intracellular signaling pathway important in regulating cell growth, differentiation, migration and survival—that are up-regulated in both human organoids and fetal cells (Jafari et al., 2019; Pollard, et al., 2006; Sánchez-Alegria et al., 2018). Pollard and et al. (2006) provided evidence that the PI3K-AKT-mTOR pathway is particularly strongly activated in the oRGCs of human organoids compared with those in chimpanzee organoids. This has been investigated further with primary human brain samples and cortical organoids, where mTOR signaling was found to play a key role in regulating the morphology and migration of human oRGCs, contributing to normal radial architecture in the human cortex (Andrews et al., 2020).

Radial glia are also reliant on NOTCH signaling, which regulates cortical progenitor proliferation and determines the final number of neurons in the mammalian cortex. When the human-specific Notch homolog NOTCH2NL was deleted in human cortical organoids, their sizes were reduced and there was premature differentiation of neuronal progenitors (Fiddes et al., 2018). This supported the role of NOTCH2NL in delaying differentiation, thereby prolonging the proliferation of cortical progenitors and increasing their neuronal production (Suzuki et al., 2018).

These types of studies will provide insight into our fundamental understanding of the mechanisms unique to humans that explain the complexity of our brains. They should also highlight, and allow the study of, human-specific molecular pathways whose disruption is likely to increase the risk of neurodevelopmental disorders. This will be particularly important for disorders such as autism spectrum disorders that affect high level cognitive functions and are difficult to model adequately in animal models, a topic we have recently reviewed elsewhere (Chan et al., 2020).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.
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