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Protocol Review

Stem Cell Models of Human Brain Development

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http://dx.doi.org/10.1016/j.stem.2016.05.022

Recent breakthroughs in pluripotent stem cell technologies have enabled a new class of in vitro systems for functional modeling of human brain development. These advances, in combination with improvements in neural differentiation methods, allow the generation of in vitro systems that reproduce many in vivo features of the brain with remarkable similarity. Here, we describe advances in the development of these methods, focusing on neural rosette and organoid approaches, and compare their relative capabilities and limitations. We also discuss current technical hurdles for recreating the cell-type complexity and spatial architecture of the brain in culture and offer potential solutions.

Introduction

Much of our current understanding of brain development and function is based upon a long history of observational and functional studies in a variety of animal models. These foundational studies have revealed general features of vertebrate and mammalian brain development that are shared across taxa, especially early events of brain patterning and neuron generation. However, specific features of human brain development and disease are much less understood. For example, of all mammals, humans exhibit the largest encephalization quotient, a measure of brain size that takes into account body size (Roth and Dicke, 2005). Furthermore, according to some measurements, the primate brain exhibits at least seven times the neuronal density of that of a rodent brain (Herculano-Houzel, 2009). Thus, the uniqueness of the human brain seems to be a product of a high neuronal density that is typical of primates, along with further expansion of overall brain size. However, the cell biological mechanisms leading to this dramatic neuronal expansion are still unclear (Geschwind and Rakic, 2013; Somel et al., 2013), and this field of research would benefit greatly from in vitro models of human brain development.

Another area of research in need of a human model system is the study of neurological disorders. Mental health disorders in particular have seen a dismal rate of new therapies in the last 40 years, which many have argued is due to problems translating findings from animals to the clinic (Matthews et al., 2005). An illustrative example of this is the psychiatric disorder schizophrenia. Many symptoms of this disorder, such a disorganized thinking, delusions, speech difficulties, and cognitive dysfunction, are difficult to examine in mice because they disrupt quintessentially human characteristics. Thus, more recent emphasis has been placed on defects at the genomic, cellular, or network level that may more likely translate from animal models to humans (Powell and Miyakawa, 2006). However, even at this level, human-specific features are evident and have been suggested to play a role in the disorder. For example, dendrite morphology in humans is more complex than in mice, with increased branching and larger spines (Defelipe, 2011), and this elaborate morphology has been demonstrated to be disrupted in both schizophrenia and autism (Penzes et al., 2011). These cellular phenotypes could be studied in in vitro human models, which would nicely complement existing animal models, the combination of which could better inform development of future therapeutics.

In this Protocol Review, we will provide an overview of the important discoveries that led to the current technologies being used to model human brain development in vitro. We will describe the key protocols, compare and contrast their strengths and weaknesses, and highlight unique features that make them important tools in this new era of human neurobiology. Finally, we will describe current technical obstacles in recapitulating in vivo phenotypes and explore possible solutions.

Overview of Mammalian Brain Development

Recent advances in understanding mammalian brain development have been essential for developing stem cell-based techniques aimed at reproducing developing brain tissue in vitro. The vertebrate brain begins as a pseudostratified epithelial sheet, the neuroepithelium (Price et al., 2011). This neural plate bends and folds to eventually close and generate the neural tube, which extends along the length of the developing embryo. The neural tube then expands laterally at varying rates along the anterior-posterior axis to give rise to various regions of the CNS, including the spinal cord, the hindbrain and cerebellum, the midbrain, and the forebrain, which in mammals gives rise to the cerebral cortical hemispheres and striatum.

Because of its epithelial nature, the neural tube is composed of apicobasally polarized neuroepithelial (NE) cells that surround a central fluid-filled lumen, which will later become the brain ventricles. After initial lateral expansion, NE cells transition to so-called radial glia (RG), which maintain their epithelial characteristics but become highly elongated as the tissue thickens (Bystron et al., 2008). The thickening of the tissue occurs as a result of the production of more differentiated cell types that migrate basally outward. These cell types are generated from self-renewing asymmetric divisions of RG which, in the mammalian cerebral cortex, can result in direct generation of neurons, or the production of intermediate transient amplifying populations that later differentiate to neurons (Figure 1). Intermediate populations include intermediate progenitors (IPs) and basal RG (bRG, or outer RG) (Fietz et al., 2010; Hansen et al., 2010; Johnson et al., 2015). bRG exhibit a relatively similar expression makeup to RG (Betizeau et al., 2013; Fietz et al., 2012; Florio et al., 2015; Pollen et al., 2015) but show heterogeneous morphologies (De

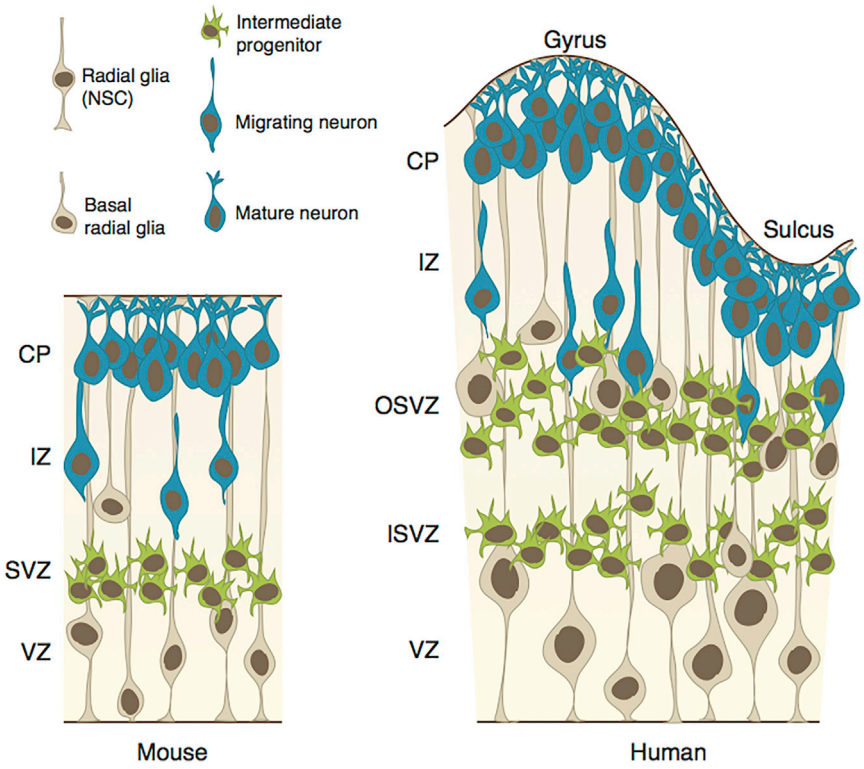

Figure 1. Schematic of Neurogenesis in the Mouse and Human Cerebral Cortex

In both humans and mice, radial glia are the neural stem cells (NSCs) that generate more differentiated daughter cells including neurons, intermediate progenitors, and basal radial glia. The radial glia possess a long basal process that attaches to the outer (basal) surface. Radial glia that reside in the ventricular zone (VZ) divide at the apical surface. Intermediate progenitors and basal radial glia reside in the subventricular zone (SVZ). In humans the SVZ is dramatically expanded with separation into an inner and outer SVZ (ISVZ and OSVZ, respectively). Neurons use the basal processes to migrate through the intermediate zone (IZ) into the cortical plate (CP). In humans, the CP is much expanded compared with mice and the cortex is highly folded with numerous gyri and sulci, whereas the mouse brain is completely smooth.

Juan Romero and Borrell, 2015), often lacking the apical connection. While RG maintain the cell body within a dense apical region called the ventricular zone (VZ), IPs and bRG translocate their cell bodies to a more basal territory termed the subventricular zone (SVZ).

Importantly, these intermediate populations (IPs and bRG) have been implicated in human brain expansion. The SVZ is generally more elaborate in larger-brained mammals such as ferrets, sheep, cats, and apes (De Juan Romero and Borrell, 2015), and it is separated into an inner and an outer SVZ (ISVZ and OSVZ, respectively). The OSVZ is completely absent in rodents (Figure 1), making it impossible to study formation of this progenitor-rich zone in mouse models. Furthermore, humans exhibit increased numbers of progenitors within the OSVZ compared with other mammals (Reillo et al., 2011), suggesting that human IPs are expanded within this zone.

Once neurons are produced, they must migrate to their proper locations. Neurons generated within the cortex rely on the long basal fascicle of the RG as a guide (Figure 1) to translocate radially (Evsyukova et al., 2013) from the VZ or SVZ through a cell-poor region termed the intermediate zone (IZ) to find their final resting place within the outer cortical plate (CP). The earliest-born neurons form an outer layer termed the preplate, which helps guide incoming neurons. Subsequent waves of neurons position themselves in an inside-out manner with the deepest layers representing earlier-born neurons while more superficial layers are composed of later-born neurons. In addition to these excitatory neurons, there are several interneuron types that modulate network dynamics in the adult brain and are thought to be key to development of a number of neurological disorders such as epilepsy, autism, and schizophrenia (Marín, 2012). These neurons are generated predominantly by the ventral forebrain and must migrate long distances tangentially around the ventricle and through the cortex to eventually integrate within the CP. Upon reaching their target location and even during migration, neurons extend axons, often over long distances, to target other neurons and begin forming a rudimentary neural network. These connections are made even before incoming stimuli and are thus intrinsically patterned, only later undergoing stimulus-driven remodeling (Sur and Leamey, 2001).

History of Neural Differentiation Methods and the Evolution of Current Techniques

Early Neural Cell Cultures

The first neural cultures were performed over a century ago. Ross Granville Harrison generated cultures from frog embryo explants in vitro and observed remarkable outgrowths of migrating neurons (Harrison, 1907). This seminal work suggested the possibility that neurons could be isolated from fetal or adult brains and cultured for in vitro examination (Millet and Gillette, 2012). Decades later, careful determination of culture conditions, media formulations, and growth factor supplementation allowed long-term culture of various neuron types, such as hippocampal, cortical, and cerebellar neurons (Brewer, 1995). While these studies allowed careful observation of neuronal function, and even network formation (Potter and DeMarse, 2001), the study of neurogenesis in vitro has been a more recent development.

Even though the term did not yet exist, neural stem cells (NSCs) were first observed by Wilhelm His at the end of the 19th century (His, 1889) with detailed descriptions of mitotic cells at the ventricular surface of the human cortex, which led him to conclude that neurons are generated at the ventricular surface and later migrate to the outer pial surface. Santiago Ramón y Cajal subsequently described the morphological features of what would later be termed radial glia (Rakic, 2003; Ramón y Cajal, 1909), and seminal work from Pasko Rakic identified the RG basal process as a guide for neuronal migration and positioning

(Rakic, 1972, 2003). We now know that His had enormous foresight, because it wasn't until the early 2000s that solid evidence proved that RG are the source of neurons and glia in the developing brain (Malatesta et al., 2000; Noctor et al., 2001; Tamamaki et al., 2001).

Sally Temple reported the first culture of isolated NSCs in 1989, showing that embryonic rat forebrain progenitors produced both neurons and glia in culture (Temple, 1989). This work began a period of intense investigation of various NSC types in vitro, isolated from different regions of both the embryonic (Lendahl et al., 1990) and adult (Reynolds and Weiss, 1992) CNS. Because of their ability to generate a variety of brain cell types, it became clear that cultured NSCs held enormous potential not only for basic discovery, but also for therapeutic possibilities (Horner and Gage, 2000). However, further careful characterization revealed that isolated NSCs in culture exhibited important differences from their in vivo counterparts, most notably an inability to recapitulate the entire range of neural lineages including intermediate amplifying populations (Conti and Cattaneo, 2010). Thus, although the therapeutic potential is still strong, the fidelity of modeling the developing brain in vivo with isolated NSCs is lacking.

As one might imagine, intact cultures of neural tissue, rather than isolated cells, have been able to better recapitulate the architecture of the developing brain. Cultures of intact CNS tissue were first performed with pieces of the embryonic chick CNS (Hoadley, 1924; Tansley, 1933; Waddington and Cohen, 1936), which displayed the remarkable ability to develop in vitro to form histologically intact early retinal tissue. Decades later, "organotypic" cultures of brain slices (Crain, 1966) became a highly valuable tool that allowed the observation of cellular behaviors within the context of a histologically intact tissue (Humpel, 2015). Such organotypic slice cultures are still heavily used today to investigate species-specific differences in brain development and neurogenesis (Lui et al., 2014).

Although slice cultures provide an important tool for descriptive analyses of brain development in a number of species, including humans, they can be difficult to acquire and the potential for functional genetic studies is limited. Instead, a system for recapitulating histologically intact tissue from individual cells would provide a tool to both perform functional studies and allow the faithful reconstruction of tissue development. The first studies of brain histogenesis from isolated cells were performed during the 1960s and 1970s when dissociation-reaggregation studies flourished (Garber, 1972; Garber and Moscona, 1972; Ishii, 1966). These experiments were performed primarily with isolated cells from embryonic chick tissues, which had previously displayed the remarkable ability to self-organize upon reaggregation and transplantation to the chick chorioallantoic membrane (Moscona and Moscona, 1952). This ability was demonstrated for a large number of tissues including kidney, liver, limb bud, skin, and various regions of the CNS. Indeed, the first description of self-organizing NE cells into tissues resembling the neural tube, so-called neural rosettes, were described in these early studies with tissue fragments (Tansley, 1933) and reaggregates (Moscona, 1957). As we will discuss, neural rosettes are now a vital member of the current toolbox of in vitro methods in developmental neurobiology. However, it took another important leap in stem cell biology to make them applicable to human neurobiology.

Neural Differentiation from Human Pluripotent Stem Cells

The derivation of the first human embryonic stem cell (ESC) lines in 1998 by Thomson et al. suddenly provided a pluripotent starting point to recapitulate embryonic development and derive a variety of tissue cell types (Thomson et al., 1998). Another leap came with the discovery of reprogramming factors to produce induced pluripotent stem cells (iPSCs), which could be generated from a variety of adult somatic cell types (Takahashi and Yamanaka, 2006). This technology opened the door to pluripotent stem cells (PSCs) from patients with genetic disorders, allowing the in vitro study of disease pathogenesis. Finally, the recent boom in genome editing technologies, such as CRISPR-Cas9 (Doudna and Charpentier, 2014), has allowed precise genetic manipulations to introduce or correct disease-associated mutations (Hockemeyer and Jaenisch, 2016), in turn allowing accurate functional analyses. It is fortuitous that these three technologies have been developed within such a short time of each other. Their convergence has provided the vital tools now allowing the generation and functional study of developing human brain tissues entirely in vitro.

Although neural rosettes were initially generated from isolated cells in early reaggregation studies (Moscona, 1957), the first derivation of human neural rosettes from ESCs was performed in 2001 (Zhang et al., 2001) (Figure 2). Zhang et al. showed that spontaneously differentiating aggregates of PSCs, called embryoid bodies (EBs), could be directed toward a neural lineage and plated on coated dishes to generate clusters of NE cells that self-organized to form rosettes. These rosette formations resemble features of the embryonic neural tube, displaying epithelial characteristics and surrounding an apical lumen. Furthermore, rosettes better recapitulate the in vivo properties of RG, as they generate intermediate populations and even a rough organization of progenitor zones similar to the VZ and SVZ (Edri et al., 2015; Shi et al., 2012). Under specific conditions, these rosettes can even be propagated and expanded (Elkabetz et al., 2008; Koch et al., 2009), an important step in functional or high-throughput studies.

Another important foundational study came from Ying et al. in 2003 (Figure 2) with the description of a protocol to differentiate ESCs to neural precursors in the complete absence of serum, growth factors, or other inductive signals (Ying et al., 2003), demonstrating the remarkable ability of PSCs to spontaneously acquire neural identities due to autocrine signaling. Modification of this method enabled the generation of dorsal forebrain progenitors capable of generating cortical neurons with the same temporal pattern as that seen in vivo (Gaspard et al., 2008).

The combination of the EB-derived rosette approach and the absence of serum described in these two earlier studies allowed the establishment of the so-called SFEB (serum free culture of embryoid bodies) method (Watanabe et al., 2005) (Figure 2). Watanabe et al. demonstrated that EBs cultured under serum-free conditions, but with the addition of specific inductive signals, could generate forebrain neural precursors when plated on coated dishes. The further development of this method, termed SFEBq, in the same laboratory allowed the formation of remarkably large rosettes when these aggregates were plated on

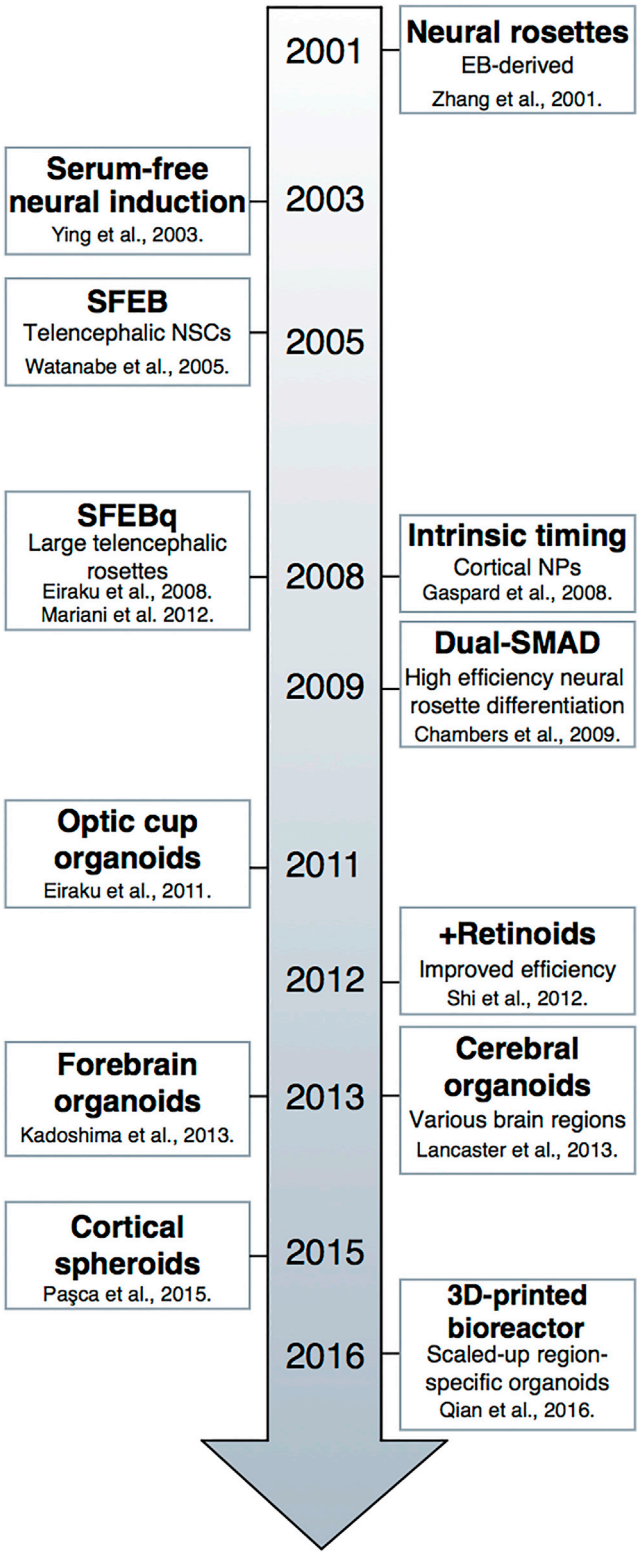

Figure 2. Timeline of Recent In Vitro Methods of Neural Differentiation

A simplified representation of the recently developed in vitro methods for the generation of neuronal tissue from human and mouse PSCs. For details of individual protocols, please see main text and Table 1.

coated dishes and allowed to differentiate further (Eiraku et al., 2008). When this method was applied to human cells in the same study, the resulting rosettes became quite large, displaying elongated lumens and thick apicobasal architecture. Indeed, because of the initially long period of 3D development, it seems that the tissues do not completely flatten upon plating. Thus, the SFEBq method pioneered and further developed by the group of Yoshiki Sasai can be thought of as existing somewhere in between 2D and 3D cultures.

The SFEBq approach has proven highly advantageous for the further development of a variety of brain regions, including adenohypophysis (Suga et al., 2011), retina (Eiraku et al., 2011), cerebellum (Muguruma et al., 2015), forebrain (Kadoshima et al., 2013), and hippocampus (Sakaguchi et al., 2015). Furthermore, this method can be applied to the study of human neurological conditions because SFEBq aggregates can be generated from iPSCs (Mariani et al., 2012). Notably, a further modification of this method with reaggregation after plating displayed large rosettes in 3D floating culture that recapitulate the developing forebrain remarkably well (Mariani et al., 2015) .

Although neural induction was previously shown to occur in the absence of serum or morphogens, the efficiency of differentiation required improvement. A large body of work, primarily in *X. laevis* embryos (Muñoz-Sanjuán and Brivanlou, 2002), has shown that germ layer specification is highly dependent on the TGFβ superfamily, which signals through a number of downstream effectors called SMADs. Non-neural identities are promoted by TGFβ members, particular Bmp and Nodal/Activin signaling (Pauklin and Vallier, 2015). Based on these observations, Chambers et al. devised an approach for efficient production of neural rosettes directly from PSCs by applying dual-SMAD inhibition to the cultures (Chambers et al., 2009), thus bypassing the need for an intermediate EB stage (Figure 1). This method was further modified with the addition of retinoids, which improved the efficiency of specification toward a forebrain identity (Shi et al., 2012).

The Development of Brain Tissues Entirely in 3D: Brain Organoids

The first entirely 3D neural culture displaying intact tissue architecture was accomplished in an elegant study by Eiraku et al. in 2011 (Figure 2) with the generation of self-organizing optic cups from human PSCs (Eiraku et al., 2011). This method built upon the previous SFEB and SFEBq methods, but by combining an altered media formulation to promote retinal identity with maintenance in floating culture rather than plating on coated dishes, the tissues that formed recapitulated the developing retina with remarkable fidelity. This study provided the first indication that neural tissue maintained in 3D floating culture could self-organize and develop histologically accurate tissue architecture.

While methods for neural differentiation from hPSCs were booming, a new field was emerging in an area of biology that at the time was quite disparate from neurobiology: the field of so-called organoids (Huch and Koo, 2015; Lancaster and Knoblich, 2014a). In a seminal study from the laboratory of Hans Clevers, Sato et al. reported that adult intestinal stem cells could generate remarkably organized 3D tissues in vitro that resembled the intestinal crypt and epithelium (Sato et al., 2009). The key discovery was the finding that embedding cells in a supportive extracellular matrix gel, called Matrigel, provided the 3D

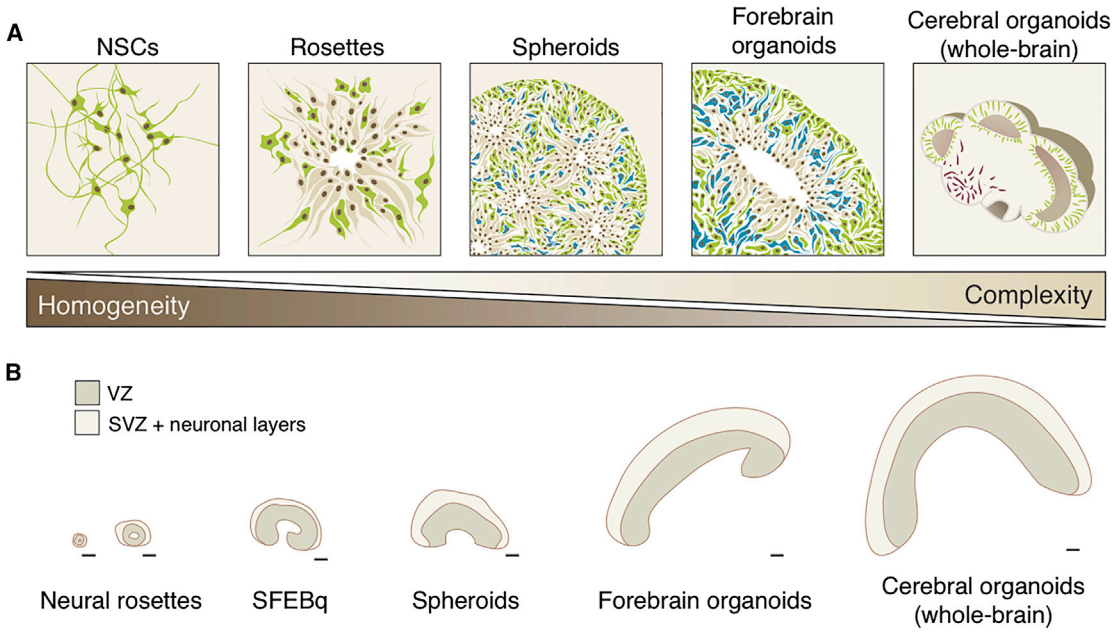

Figure 3. The Trade-Off between Homogeneity and Complexity

(A) A scale showing the relationship of the complexity of the cells/tissue produced by individual protocols and the homogeneity of the cells/tissues generated. For individual methods, please see main text. The individual cells/tissues are not shown to scale. The color scheme does not represent the exact number of cell types. (B) Cartoons depicting individual regions of cortical tissue from various methods demonstrating their relative sizes. Traces were performed on actual images from the following studies in left to right order: Neural rosettes: Figure 3d-i of Chambers et al. (2009) and Figure 1d of Kirwan et al. (2015); SFEBq: Figure 6N of Eiraku et al. (2008); cortical spheroids: Figure 2a of Paşca et al. (2015); forebrain organoids: Figure 3A of Kadoshima et al. (2013); cerebral organoids: Figure 4 of Karus et al. (2014) (this is an image of a cerebral organoid cultured as in the original Lancaster et al., 2013). Images chosen for the tracing were at similar stages of development based on timing and thickness of the VZ compared to outer regions. For those with multiple matched images, the largest was used for tracing. All images are scaled to one another; scale bar in all images represents 100 μm.

context for the self-organization of these cells into organized epithelia with typical apicobasal polarity. Subsequent studies for a variety of epithelial organs, such as stomach (Stange et al., 2013), liver (Huch et al., 2013), lung (Dye et al., 2015), and kidney (Takasato et al., 2016), have demonstrated a similar capacity for Matrigel to promote this self-organization in various contexts.

The realization that Matrigel could provide a supportive matrix for epithelia, and the fact that the brain develops as an epithelium, was an important step in the development of brain organoids (Lancaster et al., 2013) (Figure 2). Brain organoids arose from the combination of an EB approach in the absence of serum or growth factors and embedding in Matrigel. Because of the lack of inductive signals, the resulting tissues exhibited a variety of brain regional identities including hindbrain, midbrain, forebrain, and even retinal tissues. Due to this broad regionalization, these tissues were termed cerebral (from the Latin "of or relating to the brain") organoids, but here we also refer to them as whole-brain organoids to avoid confusion with region-specific tissues such as cerebral cortical or forebrain organoids. Finally, the addition of later agitation in the floating cultures promoted formation of much larger tissues than previously described. Individual organoids could reach up to 4 mm in diameter with large (>1 mm in length) continuous brain lobes (Figure 3B) containing fluid-filled cavities that resembled ventricles rather than the small neural-tube-like lumens seen in rosettes.

Likely because of their increased size, dorsal forebrain regions of cerebral organoids were also shown to exhibit highly expanded progenitor zones, even displaying a separate OSVZ, similar to human progenitor zone organization (Lancaster et al., 2013; Qian et al., 2016). Furthermore, neurons showed proper localization to the basal surface and even exhibited an organization reminiscent of an IZ and preplate, the precursor to the CP. This remarkable organization was also observed in forebrain-specific organoids generated by Kadoshima et al., who used the previously described SFEBq method with forebrain inductive signals in conjunction with continued 3D culture and addition of dissolved Matrigel (Kadoshima et al., 2013) (Figure 2). In both cerebral and forebrain organoids, the support provided by Matrigel seems to be a key factor for building structured brain elements. Matrigel has also been shown to promote formation of neuroepithelial cysts directly from human PSCs in a very short time frame which could differentiate to retinal pigmented epithelium (Zhu et al., 2013) and even patterned spinal cord (Meinhardt et al., 2014).

These whole-brain and regionally restricted organoids have recently been further modified to give rise to specific subregions (Figure 2). The combination of the strong neural inductive capability of dual-SMAD inhibition with a SFEB-type approach was recently shown to generate tissues composed of many large rosettes within a 3D context that were capable of generating the various neural and glial identities of the dorsal cortex (Paşca et al., 2015). Along these lines, Qian et al. performed dual-SMAD inhibition in combination with the cerebral organoid approach of Matrigel embedding and agitation (Qian et al., 2016), which similarly led to pure forebrain tissues, rather than

the heterogeneous regional identity of the original protocol. The authors also demonstrated the ability to derive purer midbrain and hypothalamic tissues using other combinations of inductive signals. But perhaps the most promising modification of the organoid method described in this study was the use of 3D printed mini-bioreactors for scaled-up production of neural organoids. The scale of production of brain organoids has been a limiting factor in applying this method to drug discovery, but this approach is a step toward making drug testing in regionally specified brain organoids more feasible.

Capabilities and Limitations of Current Techniques

Each of the described methods has certain limitations in addition to their many benefits. When deciding which method to use, one must consider multiple variables such as technical considerations, the timescale required for the particular method, and the specific biological question. We will consider each of these aspects for the three major approaches described here: 2D neural rosettes, organoids or spheroids for individual brain regions, and whole-brain organoids.

Technical Considerations

While all three approaches are feasible in most tissue culture (TC) laboratories, some require more specialized equipment or complicated culture conditions. The first major hurdle for many labs interested in using these techniques is establishing human PSC culture as a routine practice. Human PSCs are notoriously finicky, but improved protocols, for example feeder-free approaches (Chen et al., 2014; Ludwig et al., 2006), have made the process much less laborious. Nonetheless, a laboratory already proficient in human PSC culture can more easily implement any of the described methods, often with minimal equipment costs and low activation energy.

Rosettes require the least in terms of equipment as they can be grown with standard TC equipment and good sterile culture technique (Table 1). Particularly the dual-SMAD direct differentiation approaches (Chambers et al., 2009; Shi et al., 2012) are quite straightforward and have a high efficiency of generating neural rosettes. Furthermore, these methods do not contain any 3D steps and therefore do not require special low-attachment dishes, or manual isolation of rosettes, unlike EB-derived rosettes (Elkabetz et al., 2008; Koch et al., 2009; Zhang et al., 2001).

3D tissues require more specialized TC conditions (Table 1) and in order to maintain their 3D nature, these tissues must be grown on low-attachment plates or dishes (Eiraku et al., 2008). Furthermore, the majority of those methods that maintain the culture entirely in 3D even at later stages require a method to increase oxygen exchange. This can be accomplished by culturing in a high oxygen environment (40%), which requires a special incubator and access to O_2 lines (Eiraku et al., 2008; Kadoshima et al., 2013; Sakaguchi et al., 2015), or by agitation, either in a spinning bioreactor or orbital shaker (Lancaster et al., 2013; Lancaster and Knoblich, 2014b; Qian et al., 2016). This requires equipment in addition to the standard TC set up. Finally, many 3D methods also require manual steps such as the dissection of regions in the retinal organoid approach (Eiraku et al., 2011), the manual isolation and aggregation culture of rosettes that was recently developed (Mariani et al., 2015), and the embedding of cerebral organoids in Matrigel droplets (Lancaster et al., 2013; Lancaster and Knoblich, 2014b). These can be technically challenging, but are typically feasible with practice.

Timing

As a rule, differentiation protocols are much accelerated when performed with mouse PSCs compared with human PSCs (Table 1). The dramatic difference in timing between human and mouse can be seen in the finding that, regardless of method, mouse cells generate polarized NE cells within 4–5 days (Eiraku et al., 2008) and the first neurons are generated as early as 5 days after beginning differentiation (Ying et al., 2003). In contrast, methods with human cells require 7–10 days to generate NE cells (Chambers et al., 2009; Zhang et al., 2001) and the first neurons are not visible until approximately 20 days (Lancaster and Knoblich, 2014b; Shi et al., 2012). This timing does not seem to be dependent on method as this trend is seen in both 2D and 3D methods. Instead, there seems to be strong intrinsic time keeping of the cells during developmental events. Indeed, when transplanted into the mouse brain, human PSC-derived neurons exhibited protracted maturation despite their surrounding (Espuny-Camacho et al., 2013; Maroof et al., 2013; Nicholas et al., 2013), indicating a cell-intrinsic timing reflecting human neurodevelopmental neoteny.

Thus, neural tissues can be generated much faster with mouse PSCs than human PSCs. However, often the use of human PSCs is desirable particularly for modeling human-specific features or utilizing patient-derived iPSCs. The main consideration when contemplating timing of human-derived methods is what stage of neural development is most important for the question at hand. For example, studies of tissue patterning could be performed as early as 10–15 days, while neuronal studies must be performed after 30–40 days, and even up to 100 days in the case of synaptic maturation (Kirwan et al., 2015). This timing is quite comparable to that seen in the human fetal brain, where neural induction begins at day 12 with primitive streak formation, neurogenesis begins to escalate at 40–50 days, and synaptogenesis begins at approximately 80–90 days (Silbereis et al., 2016). Furthermore, expression analyses have demonstrated the ability of a variety of methods to recapitulate first trimester human fetal brain development (until 8–10 weeks of gestation) (Camp et al., 2015; Mariani et al., 2012; van de Leemput et al., 2014) and even up to mid-gestation (19–24 weeks gestation) (Paşca et al., 2015; Qian et al., 2016).

The Trade-Off between Homogeneity and Complexity

Current methods for human neural differentiation in vitro span a wide range of complexity (Figure 3A). At one end of the spectrum, monolayers of unpolarized NSCs are the least complex, but most homogeneous (Pollard et al., 2006). These cells represent a fairly pure population of a single identity that has limited differentiation potential. However, because of their homogeneity, these NSCs are a useful system for high-throughput screening (Garavaglia et al., 2010; Kim et al., 2012).

Neural rosettes are also cultured in 2D but with increasing complexity. Because these are polarized epithelial cells, they self-organize to form a characteristic radial arrangement. This arrangement allows for better recapitulation of neurodevelopmental events with intermediate populations that even migrate to the basal edge of the rosette, producing a zone reminiscent of the SVZ (Shi et al., 2012). The strong interdependence of structure and function is demonstrated here by the fact that

Table 1. Overview of Current Methods

Result	EB Step?	Growth Factors or Inhibitors[a]	Entirely 3D?	Protocol Overview	Technical Requirements[b]	Timing[c]	Reference
Neural rosettes	yes	FGF2	no	ESC → EBs → plating → rosettes	manual isolation	7 days	Zhang et al., 2001
Neural progenitors (NPs)	no	none	no	ESC → NPs	none	4 days§	Ying et al., 2003
Forebrain NPs	yes	Wnt inh., Nodal/Act/TGFβ inh.	no	ESC → EBs → plating → forebrain NPs	none	10 days§	Watanabe et al., 2005
Propagating rosettes	yes	FGF2, SHH, FGF8, AA, BDNF	no	ESC → EBs → plating → rosettes → propagation	manual isolation	12–16 days	Elkabetz et al., 2008
Cortical NPs	no	cyclopamine	no	ESC → cortical NPs	none	10–14 days	Gaspard et al., 2008
Large forebrain rosettes	yes	Wnt inh., Nodal/Act/TGFβ inh., BMP inh.	no	ESC → EBs → plating → forebrain rosettes	none	10 days§, 46 days	Eiraku et al., 2008
Neural rosettes	no	Nodal/Act/TGFβ inh., BMP inh.	no	ESC → neural rosettes	none	11 days	Chambers et al., 2009
Propagating rosettes	yes	FGF2, EGF	no	ESC → EBs → plating → rosettes → propagation	manual isolation	8–15 days	Koch et al., 2009
Retinal organoids	yes	none	yes	ESC → EBs → optic cup → retinal organoids	manual dissection, 40% O_2	24 days§	Eiraku et al., 2011
Adenohyphysis organoids	yes	Hh agonist, additional depending on endocrine types	yes	ESC → EBs → Rathke's pouch pituitary progenitors	40% O_2	21–33 days§	Suga et al., 2011
Neural rosettes	no	Nodal/Act/TGFβ inh., BMP inh., retinoids	no	ESC → neural rosettes	none	15 days	Shi et al., 2012
Large forebrain rosettes	yes	FGF2, Wnt inh., Nodal/Act/ TGFβ inh., BMP inh.	no	iPSC → EBs → plating → forebrain rosettes	none	45–50 days	Mariani et al., 2012
Neuroepithelial cysts	no	none	yes	ESCs → NE cysts → retinal pigment epithelium; or spinal chord	none	5 days	Zhu et al., 2013; Meinhardt et al., 2014
Whole-brain organoids	yes	none	yes	ESC/iPSC → EBs → Matrigel embed → agitation → brain organoids	manual embedding, agitation	30–40 days	Lancaster et al., 2013
Forebrain organoids	yes	Nodal/Act/TGFβ inh., Wnt inh.	yes	ESC → EBs → forebrain organoids	40% O_2	42 days	Kadoshima et al., 2013
Cerebellar organoids	yes	Nodal/Act/TGFβ inh., FGF2, FGF19, SDF1	yes	ESC → EBs → cerebellar organoids	none	35 days	Muguruma et al., 2015
Cortical spheroids	yes	Nodal/Act/TGFβ inh., BMP inh., FGF2, EGF, BDNF, NT3	yes	iPSC → EBs → cortical spheroids	none	43 days	Paşca et al., 2015
Aggregates of large forebrain rosettes	yes	BMP inh., Wnt inh., FGF2, EGF	no	iPSC → EBs → plating → forebrain rosettes → floating aggregates	manual isolation	42–44 days	Mariani et al., 2015
Hippocampal-Choroid plexus organoids	yes	Wnt inh., Nodal/Act/TGFβ inh., Wnt(CHIR), BMP4	yes	ESC → EBs → Hippocampal-Choroid plexus organoids	40% O_2	35–42 days	Sakaguchi et al., 2015
Forebrain, midbrain, or hypothalamic organoids	yes	Nodal/Act/TGFβ inh., BMP inh., additional depending on region	yes	iPSC → EBs → Matrigel embed → scaled-up agitation → regional organoids	manual embedding, 3D printed agitation in multi-well	28 days	Qian et al., 2016

Methods are listed chronologically. Inh., inhibition; Act, Activin; AA, ascorbic acid.
[a]Factors in neural induction or regional specification are listed.
[b]Technical requirements beyond standard sterile TC culture.
[c]Timing from human PSCs (except §, which indicates mouse PSC timing) to achieve the result listed in the table.

NSCs in this context have increased differentiation capacity and can better recapitulate the lineage of the developing brain.

The SFEBq-based methods developed by the Sasai group represent a substantial elaboration of 2D rosette methods, and they can be rather thought of as 2.5D. Although these are plated on coated dishes, the tissues do not entirely flatten and maintain a high level of complexity with more extended apical surfaces (Figure 3B). The derived tissues exhibit an architecture that is remarkably similar to the early forebrain, displaying organized progenitor zones. In contrast to neural rosettes, these zones are well maintained over time and neurons remain within the tissue rather than migrating away throughout the dish.

3D methods are at the far end of the spectrum; they are highly complex, but also more heterogeneous compared with 2D methods (Figure 3A). The spontaneous nature of their self-organization results in variability in morphology between various areas of the tissue (Eiraku et al., 2011; Lancaster et al., 2013) and between different batches (Lancaster and Knoblich, 2014b). Despite this heterogeneity, individual regions show expanded tissue architecture not seen with 2D methods (Figure 3B). However, high heterogeneity inherently leads to issues with reproducibility, making it difficult to perform high-throughput screening with a robust and reliable readout. Thus, these methods are typically utilized for testing specific hypotheses where careful morphological analyses can be performed.

For example, such an approach has been applied to the study of microcephaly (Gabriel et al., 2016; Lancaster et al., 2013), revealing a dramatic reduction in overall organoid size and reduced neurons and progenitors. Further analyses demonstrated premature neuronal differentiation and depletion of RG progenitors. More recently, forebrain tissues based on the SFEBq approach have been utilized for the study of idiopathic autism (Mariani et al., 2015). In this case, forebrain aggregates derived from patient iPSCs displayed overproduction of inhibitory interneurons. Finally, very recent studies with brain organoids have revealed a strong effect of Zika virus infection on neural progenitor survival (Cugola et al., 2016; Dang et al., 2016; Garcez et al., 2016; Qian et al., 2016). In all of these examples, the phenotypes were quite dramatic and importantly lied outside the normal phenotypic range of such 3D methods. Furthermore, previous animal model and clinical studies had hinted at potential mechanisms that could be examined in organoids, allowing the targeted analysis and identification of these phenotypes. Thus, in the context of disease pathogenesis, 3D organoid approaches are currently most useful for testing specific hypotheses underlying strong phenotypes.

The heterogeneity of organoids is not only dependent on the 3D nature of the culture method. In fact, the degree to which the resulting tissue is restricted to a particular regional identity is likely more influential. For example, the recently described cortical spheroids are cultured entirely in 3D and yet display remarkably homogeneous morphology with multiple cortical rosette-like structures that are highly similar within a single spheroid (Paşca et al., 2015). Instead, whole-brain organoids exhibit the highest level of complexity described to date, with highly expanded regions of different identities (Lancaster et al., 2013). However, identity determination varies from experiment to experiment, and neural induction is not always complete, with occasional non-neural identities forming (Camp et al., 2015). This is likely due to the lack of exogenous inductive growth factors. Furthermore, brain regional identity appears spontaneously, leading to stochastic formation of various regions in configurations that are unique to each individual organoid.

Yet this patchwork of brain regions seems to be beneficial for tissue architecture. For example, tangential migration of interneurons from ganglionic eminence tissue to dorsal cortex has been reported within a single whole-brain organoid (Lancaster et al., 2013), which importantly recapitulates communication between these brain regions. Furthermore, neurons within organoids can extend very long axons from one region to another within the organoid, suggesting the intriguing possibility that intrinsically patterned connectivity, such as that seen in the intact developing brain, may be recapitulated in this context.

The balance between complexity and heterogeneity is an important feature to consider when deciding which method to use for a particular study (Figure 3A). A more homogeneous system would be needed for screening approaches, and making neural differentiation methods even more homogeneous will be necessary to translate them to drug testing paradigms. 3D methods are instead more suited to hypothesis testing that requires a more accurate representation of the tissue. However, these approaches can be used to perform disease modeling in a more efficacious manner because targets of interest are in their physiologically relevant context. Finally, the even more complex whole-brain organoids are perhaps most suited to studies requiring the presence of various brain regions, such as long-range neuronal migration, or network formation between brain regions. An ideal model system would be both complex in its morphology and reproducible, thus coming as close to possible to the in vivo situation. As we will discuss, perhaps future technological advances will enable this ideal model (Figure 4).

Future Outlook: Technical Strategies to Advance Models of Human Brain Development

Current Technical Obstacles

While individual methods develop different repertoires of brain regional identities, they all have a similar technical hurdle, namely the lack of overall spatial patterning of a developing brain in vivo. None of the methods generate tissues that have the overall shape of a developing human brain, and this is likely due to the lack of embryonic body axes that would normally guide the directionality of brain development. Whether in whole-brain organoids or isolated forebrain organoids, there are no anterior-posterior or dorsal-ventral axes that remain continuous through the organoid and pattern across the tissue. This is a serious limitation, not only with regard to recapitulation of events in vivo, but also because this deficiency is largely responsible for the heterogeneity and variability from organoid to organoid. Whereas in vivo, any two brains look quite similar even from the outside, two organoids do not. This heterogeneity can also prove challenging during analysis, as extra care needs to be taken in order to ensure that the desired brain region is actually present in a given organoid.

A second limitation concerns later neural tissue maturation, and particularly the organization of neurons as they reach the basal surface of cortical tissues. While several 3D and 2.5D methods

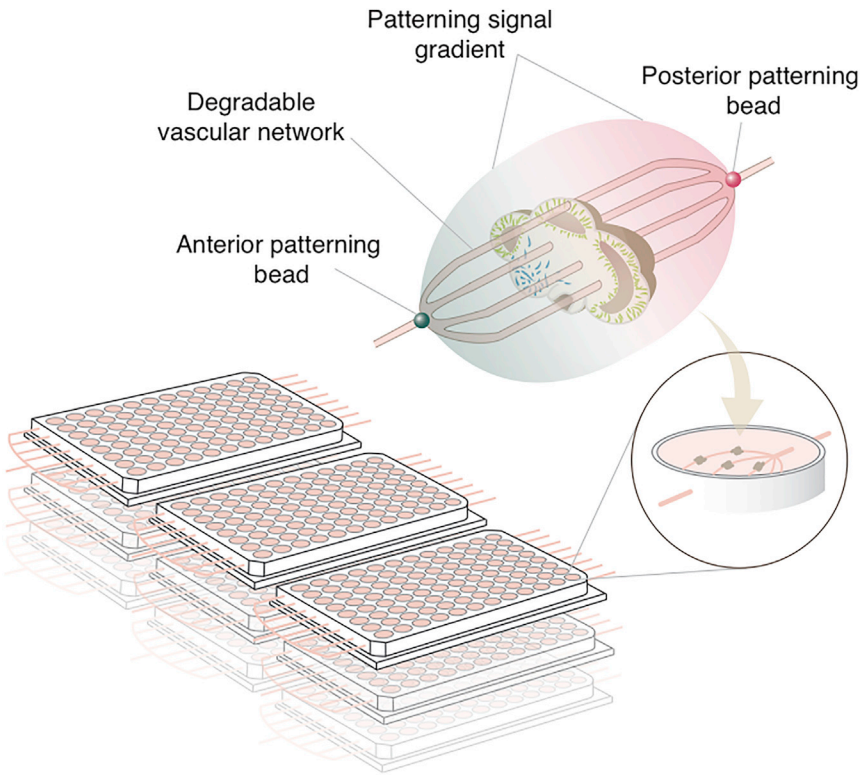

Figure 4. High-Throughput Engineering of Vascularized Organoids

A cartoon showing the potential technical improvements that would lead to highly reproducible brain organoid cultures on a large scale. Organoids might be grown in multi-well dishes or some other large-scale system. Vasculature-like network might be achieved by using degradable networks for the constant supply of oxygen and nutrients. Axial patterning could be accomplished with the usage of patterning beads, either incorporated into the organoid or as a part of the scaffold.

have described remarkable morphologies of cortical progenitor zones (Eiraku et al., 2008; Kadoshima et al., 2013; Lancaster et al., 2013; Mariani et al., 2015; Paşca et al., 2015), neurons fail to generate key structures in cortical development. Specifically, in vivo, neurons initially form a disorganized layer on the outer surface called the preplate. However, subsequently born neurons migrate into the preplate and cause it to split into the marginal zone and the subplate, positioning themselves in a dense, radially aligned band. This band is the early CP, and over time it thickens with the addition of new neurons and forms the layers characteristic of the cerebral cortex. The initial splitting and condensation of the CP has been shown to be necessary for subsequent layer formation in vivo (Olson, 2014; Rakić et al., 2006), while Cajal-Retzius cells in the resultant marginal zone are necessary for proper inside-out layer formation and the development of six distinct layers (Frotscher, 1998).

Remarkably, several 3D methods have demonstrated at least rudimentary separation of early-born and late-born neurons into deep and superficial layers (Kadoshima et al., 2013; Lancaster et al., 2013; Paşca et al., 2015; Qian et al. 2016). Perhaps the most dramatic illustration of this was seen in cortical spheroids (Paşca et al., 2015) and forebrain organoids (Qian et al. 2016), which showed a clear segregation of deep and upper layer neurons into separate zones. This is striking given that this seemed to occur in the absence of a marginal zone or subplate and without preplate splitting and CP condensation. The lack of these supportive zones, and even a lack of an IZ, raises questions as to how these neurons are able to self-organize into discrete regions. It will be important to further examine the development of these 3D systems with particular attention to the radial glial scaffold, the presence of guidance cues, and later potential separation into six discrete layers. Because neuronal connectivity is determined by relative position within these layers (Harris and Mrsic-Flogel, 2013), recapitulation of this organization would be important in future modeling of network formation.

A final major limitation, for 3D models specifically, is the inadequate supply of nutrients and oxygen to the central regions of the tissue. Because cells farther than 200–400 μm from the surface of brain tissue fail to receive enough nutrients through diffusion (Rambani et al., 2009), healthy tissue is limited to the surface of organoids (Lancaster et al., 2013). This has effects on everything from overall tissue patterning to later expansion of individual brain regions. Indeed, this may contribute to the observation in 3D systems of deep and superficial zones, but not six discrete layers. In the human cortex, these layers span over 2 mm (Defelipe, 2011), a length well beyond the diffusion limit. Thus, the lack of nutrients deep within the tissue will be an important hurdle to overcome in the future.

Expectations for Future Technological Advances

We expect that the years to come will see a coming together of two fields: tissue engineering and organoids (Yin et al., 2016). Likely this will impact organoids for a variety of organs, but specifically for the brain, such a synergy could provide significant advantages. With regard to shape and the lack of body axes, scaffolds could provide the necessary structure to pattern the organoids and at the same time deliver morphogens in a spatially restricted manner (Carlson et al., 2016; Langer and Vacanti, 2016; Pan and Ding, 2012). Alternatively, beads releasing patterning factors could be implanted into organoids to provide these cues (Lee et al., 2011). In this way, external body axes could be provided that would help shape the organoid and reproducibly generate continuous brain regions across the organoid (Figure 4).

Another future advance could include co-cultures of brain organoids with supportive non-neural types. In vivo, later neural development relies on increasing numbers of extra-CNS cell types. Initially, the brain develops without vasculature, but at around 3 weeks of development (Bauer et al., 1992), blood vessels begin to penetrate the developing brain from the overlying pia, or meninges, a non-neural membrane that provides not only vasculature but also the basement membrane to which

RG attach their basal processes. This attachment is thought to be key to the maintenance of the radial glial scaffold for proper neuronal migration (Haubst et al., 2006). Thus, this key non-neural membrane is vital for both vascularization and neuronal migration. Perhaps ectopic co-cultures of meninges with brain organoids would not only support the RG scaffold, but also allow vascularization of brain organoids.

The lack of vascularization has been an issue for the field of tissue engineering even before the first organoids were established (Rouwkema et al., 2008). As a result, bioengineers have devised inventive approaches to mimic vascular networks entirely in vitro (Auger et al., 2013). For example, endothelial cells can be cultured in microfluidic channels and will line the inner surface of the channels just as they would within a blood vessel (Schimek et al., 2013; van Duinen et al., 2015). Furthermore, microfluidic channels made from degradable materials, such as collagen gels, allow vascular remodeling and the growth of new vessels de novo (Rouwkema and Khademhosseini, 2016). Perhaps the co-culture of an organoid on such a degradable vascular network would allow angiogenesis of the organoid completely in vitro (Figure 4).

Although co-cultures with vascular cells, either derived from meninges or as individual endothelial cells, may be capable of forming tubular networks within organoids, there remains the issue of nutrient supply. Without a source of blood or blood-substitute as well as a means to pump the fluid through the network, the tissue would still suffer from nutrient and oxygen depletion. Thus, an alternative approach may be transplantation into an animal host. Ectopic transplantations have been performed with liver organoids with quite high success (Takebe et al., 2013). This powerful combination of an in vitro human organoid and an in vivo animal environment led to perfused tissues that were able to secrete factors into the host blood stream. Perhaps such an ectopic transplantation approach would enable brain organoids to mature to later stages of neural development when questions regarding neuronal migration or maturation could be examined.

Finally, perhaps the greatest application of these in vitro methods will be for disease modeling and drug discovery. However, the current scale of production, particularly for 3D organoids, is highly limiting and reproducibility remains an issue. Reliable disease modeling and identification of drug-induced phenotypes will require both highly reproducible and scaled-up systems. Notably, both these issues have very recently been addressed with the work from Qian et al. demonstrating the use of 3D printed bioreactors that can easily be scaled up (Qian et al., 2016). The combination of higher throughput with dual-SMAD inhibition leads to reproducible forebrain organoids that hold great promise for future therapeutic avenues.

Conclusions

Modern developmental neurobiologists are in the fortuitous position of doing research at a time of rapid technological advancement. As a result, extraordinary progress has been made in recent years in the development of in vitro models of human brain development. Since the first neural 3D system was described for the retina 5 years ago (Eiraku et al., 2011), at least eight new neural organoid methods (Table 1) have been developed, ranging from isolated brain regions (i.e. cortical spheroids; Paşca et al., 2015) to larger regional identities (forebrain organoids; Kadoshima et al., 2013; Mariani et al., 2015) and whole-brain organoids (Lancaster et al., 2013). While these methods span a range of complexity, they also display an opposing range of homogeneity and reproducibility. This trade-off is an important consideration for researchers contemplating using these approaches, and the choice depends highly on the scientific question at hand. However, we are hopeful that future endeavors will enable 3D model systems that recapitulate both the complexity of the human brain and its reproducible formation. If this can be achieved, such a system would provide a relevant model that could be used for a broad array of studies. These include basic neurobiological studies of human neurodevelopment and evolution, as well as the study of neurological and mental health disorders, such as schizophrenia and autism. Furthermore, recent findings with Zika virus open the door to a variety of potential studies of other infectious agents such as cytomegalovirus. The hope is that one day, these approaches will complement existing animal studies to enable targeted design of novel therapeutic drugs.

CONFLICTS OF INTEREST

M.A.L. is an inventor on patent applications describing the development of cerebral organoid methods.

ACKNOWLEDGMENTS

The authors would like to thank members of the Lancaster laboratory as well as Mario DeBono, Davide Gianni, Veronica Krenn, Joshua Bagley, and Juergen Knoblich for helpful comments on the manuscript. Work in the Lancaster laboratory is supported by the Medical Research Council MC_UP_1201/9 and a 3Rs prize from the National Centre for the Replacement, Refinement and Reduction of Animals in Research.

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