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## **Modeling Genetic Epilepsies In A Dish**

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## **ABSTRACT**

Human pluripotent stem cells (hPSCs), including embryonic and induced pluripotent stem cells, provide a powerful platform for mechanistic studies of disorders of neurodevelopment and neural networks. hPSC models of autism, epilepsy and other neurological disorders are also advancing the path toward designing and testing precision therapies. The field is evolving rapidly with the addition of genome-editing approaches, expanding protocols for the two-dimensional (2-D) differentiation of different neuronal subtypes, and three-dimensional (3-D) human brain organoid cultures. However, the application of these techniques to study complex neurological disorders, including the epilepsies, remains a challenge. Here we review previous work using both 2-D and 3-D hPSC models of genetic epilepsies, as well as recent advances in the field. We also describe new strategies for applying these technologies to disease modeling of genetic epilepsies, and discuss current challenges and future directions.

## **Introduction**

Epilepsy is one of the most common neurological disorders with a prevalence in the U.S. of 1.2% (Zack and Kobau, 2017). The hallmark feature of epilepsy is spontaneous recurrent seizures, but the disease encompasses a wide range of etiologies, behavioral manifestations, electrographic signatures, pharmacological profiles and histopathologies (Grone and Baraban, 2015). Epilepsy also has a high incidence early in life and is associated with increased mortality. Approximately 30-40% of patients cannot be adequately controlled with anti-epileptic drugs (Brodie et al., 2012). Only a small portion of medically refractory patients are surgical candidates, but most of these candidates never receive an evaluation for resective brain surgery. Unfortunately, no therapies exist for preventing epilepsy that may occur from various causes, such as after head trauma, stroke or other neurological insult, from a genetic predisposition, or a malformation of brain development. Overall, epilepsy poses a huge burden to patients and their families.

The number of genes associated with epilepsy has exploded since the first gene linked to a clinical seizure phenotype was discovered nearly three decades ago (Shoffner et al., 1990). To date, over 500 loci are linked to epilepsy (Noebels, 2015), but our understanding of how these mutations

lead to spontaneous seizures and other epilepsy manifestations lags behind gene discovery. Traditional strategies to model genetic epilepsies have involved the use of rodent models, obtained either through genetic modification or finding spontaneous mutations within rodent colonies. Despite many advances made studying animals, these models do not fully recapitulate human disease phenotypes and mechanisms, likely because human brain development differs considerably from that of the rodent brain in many critical aspects (Zeng et al., 2012; Rakic, 1995; Miller et al., 2019). In addition, many genetically modified mouse models of epilepsy fail to show spontaneous recurrent seizures (the hallmark of epilepsy) and may only exhibit signs of cortical network excitability through testing of seizure thresholds after chemoconvulsant administration. Limited scalability of rodent models also constrains the possibility of performing rapid mutation-specific mechanistic studies and drug screening. Postmortem tissues or surgical specimens from patients are difficult to acquire and those available generally represent late-stage disease, offering limited insight into causative mechanisms. Zebrafish models of genetic epilepsies are gaining traction and are extremely amenable to high-throughput drug screening (Griffin, 2016). The fish models have only been useful for interrogating loss-of-function epilepsy genes, although recent advances in gene editing technologies may allow for epilepsy missense mutations to be interrogated in zebrafish models in the future (Prykhozij et al., 2018). Consequently, decades of research have not produced profound mechanistic insights into any of the many epilepsy syndromes, including the monogenic epilepsies.

The derivation of human embryonic stem cells (hESCs) from blastocysts by the Thomson laboratory in 1998 (Thomson et al., 1998) and the generation of mouse induced pluripotent stem cells (iPSCs) through somatic-cell reprogramming by Yamanaka and colleagues in 2006 (Takahashi and Yamanaka, 2006) opened the door for translational research using stem cells in disease modeling and therapeutic development. The discovery of mouse iPSCs was quickly followed by the generation of human iPSCs (Takahashi et al., 2007; Meissner et al., 2007). Soon after, iPSC disease modeling was developed by differentiating the cells into tissues relevant for investigating specific diseases (Dimos et al., 2008; Park et al., 2008). Patient iPSCs carry the identical mutation(s) in the context of the patient's specific genetic background. Given the inability to acquire disease tissue for *in vitro* study from a human subject with a central nervous system (CNS) disorder during the subject's life,

the differentiation of hPSCs to neural cells or brain-like structures offers an unprecedented opportunity to model human brain development and brain disorders.

Various protocols have been developed to differentiate hPSCs to neural cells that are most relevant to models of epilepsy, such as cortical-like excitatory neurons (Chambers et al., 2009; Shi et al., 2012a), cortical-like inhibitory neurons (Liu et al., 2013a; Maroof et al., 2013; Nicholas et al., 2013), dentate granule cell-like neurons (Yu et al., 2014) and astrocytes (Williams et al., 2014). These neurons in 2-D cultures form functional synapses and eventually neuronal networks. To better recapitulate the architecture of the developing human brain, efforts have been made to generate 3-D cerebral organoids from iPSCs (Eiraku et al., 2008; Lancaster et al., 2013). Some of these brain organoids have features that resemble cerebral cortex, ventral telencephalon and also hippocampal anlage (Lancaster and Knoblich, 2014a; Lancaster et al., 2013). More recent advances include the development of more uniform and defined brain region-specific organoids/spheroids (Birey et al., 2017; Pasca et al., 2015; Qian et al., 2016; Watanabe et al., 2017; Xiang et al., 2019; Xiang et al., 2017). Progress in neural differentiation protocols also makes iPSCs very appealing for stem cell-based transplantation therapies for neurological disorders, an area that is being actively studied (Hargus et al., 2010; Lu et al., 2014; Oki et al., 2012; Wang et al., 2013b; Mansour et al., 2018a). The application of genome editing to hPSCs further enhances the utility of *in vitro* human cell models of epilepsy. Genome editing technologies, such as TALEN (transcription activator-like effector nuclease) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 nuclease) systems can be used to modify hPSCs directly (Miyaoaka et al., 2014; Soldner et al., 2011), or simultaneously with fibroblast reprogramming (Howden et al., 2015; Tidball et al., 2017). This approach is particularly useful given the limited access to subjects with rare epilepsy mutations, or to study the effects of specific mutations in an isogenic control background.

To date, iPSCs have been generated for well over two dozen different CNS disorders (Marchetto et al., 2011; Srikanth and Young-Pearse, 2014) and the number continues to grow rapidly. hPSCs have been used to model a wide array of neurological disorders with nearly completely penetrant epilepsy phenotypes, such as Dravet syndrome (DS) (Liu et al., 2013b; Sun et al., 2016b), Angelman syndrome (AS) (Chamberlain et al., 2010), variant Rett syndrome caused by cyclin-dependent kinase-like 5 (*CDKL5*) mutations (Ricciardi et al., 2012; Livide et al., 2015) and Syntaxin-

Binding Protein 1 (STXBP1)-Related Epileptic Encephalopathy (Yamashita et al., 2016; Patzke et al., 2015). This approach has also been employed to model other neurodevelopmental disorders with seizures as one of the comorbidities, including Tuberous Sclerosis Complex (TSC) (Li et al., 2017a; Zucco et al., 2018; Blair et al., 2018; Nadadhur et al., 2019; Sundberg et al., 2018), typical Rett syndrome (Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Marchetto et al., 2010; Mellios et al., 2018), Fragile X syndrome (Liu et al., 2012; Sheridan et al., 2011; Urbach et al., 2010), Timothy syndrome (Krey et al., 2013; Pasca et al., 2011), 15q11.2 deletion syndrome (Yoon et al., 2014), Phelan-McDermid syndrome (Shcheglovitov et al., 2013), and Miller-Dieker Syndrome (Bershteyn et al., 2017). More recently, hPSC cultures have been used to study microcephaly resulting from ZIKV exposure (Li et al., 2017b). Although whole animal models still remain critical for studying various behavioral and electrophysiological features of human epilepsies, "epilepsy in a dish" models are becoming critical platforms to study disease mechanisms and to develop patient-specific therapeutic interventions (EpiPM Consortium, 2015; Parent and Anderson, 2015). Below we review recent advances in hPSC technology (with a focus on genome editing) and iPSC-derived 2-D and 3-D neural culture methods, as well as their applications for disease modeling. Finally, we discuss the challenges and potential future directions of these model systems.

### **Generation of iPSCs**

The generation of patient-specific iPSC lines enables a wide repertoire of genetic epilepsies to be modeled in culture. iPSCs are generated by the forced expression of specific transcription factors in somatic cells (Takahashi and Yamanaka, 2006), a process called "reprogramming," to reset the epigenetic state of the cells similar to the pluripotent state of hESCs derived from pre-implantation human embryos. Most often, the reprogramming is performed on dermal fibroblasts isolated from a skin biopsy (Vangipuram et al., 2013), but more recently hematopoietic cells (Okita et al., 2013; DeRosa et al., 2012) or kidney epithelial cells derived from urine (Zhou et al., 2011; Wang et al., 2013a) have been used. iPSCs can self-renew and have the potential to generate cells in the three germinal lineages and their subsequent progeny. Therefore, they provide a potentially unlimited source of starting material from which they can be differentiated into various cell types for disease modeling, drug screening, toxicity studies or regenerative therapies.

The utility of iPSCs can be further expanded by genome editing, particularly using the CRISPR/Cas9 system, which can make precise changes in cellular genomic DNA (Cong et al., 2013; Ran et al., 2013; Jinek et al., 2013; Mali et al., 2013) or can be used to modulate gene expression (Gilbert et al., 2014). This two-component system consists of a Cas9 nuclease and a single guide RNA (sgRNA) that mediates DNA binding specificity. In brief, Cas9 nuclease is targeted to a genomic DNA sequence of interest where it introduces a double-strand break (DSB). The DSB triggers the endogenous DNA repair machinery to repair the break *via* one of two pathways: an error-prone non-homologous end-joining (NHEJ) pathway or a homology directed repair (HDR) pathway (Featherstone and Jackson, 1999). The NHEJ pathway joins DNA ends in a manner that often introduces random insertion or deletion (indel) mutations (Lieber, 2010). These indels can introduce frame shifts and premature stop codons when targeted to open reading frames, thus disrupting gene function through nonsense-mediated mRNA decay (NMD) mechanisms (Cho et al., 2013). Alternatively, when the HDR pathway is activated in the presence of a donor template with sequence homology to the region flanking the DSB (Jasin and Rothstein, 2013), the breaks can be repaired seamlessly with insertion of the donor nucleotides. Changes of either single nucleotide or large exogenous DNA sequences can be introduced into the genomic region of interest when it is included in the repair template.

Using CRISPR/Cas9 genome editing, any gene of interest, in theory, can be deleted or altered to interrogate its role in biological processes, while circumventing difficulties in obtaining patient tissues and allowing for comparison with an isogenic control (Santos et al., 2016). Loss of function (LOF) “virtual patient” lines can be generated through the NHEJ pathway. Patient-specific mutations, including inherited or *de novo* germline mutations, can also be edited into a control iPSC line through HDR-mediated repair when a template carrying the mutation is provided. Generating additional “virtual patient” lines from the same genetic background also allows for direct comparisons among specific patient mutations (Miyaoka et al., 2014). However, HDR-mediated repair is less efficient than NHEJ. The repair efficiency can vary by an order of magnitude among cells of different types or even those of the same type but of different genetic backgrounds (Miyaoka et al., 2016). Moreover, a potential limitation of using “virtual patient” lines in a control iPSC line is the loss of patient-specific genetic modifiers. Therefore, combining this approach with patient-specific material is often ideal for

confirming cellular phenotypes associated with specific mutations. Alternatively, CRISPR/Cas9 based genome editing may be used to reverse a genetic variant of interest to the wild-type status (Schwank et al., 2013). Since genotypic variability is a major driver of phenotypic variability, isogenic controls are vital to determine causality between genotype and phenotype. Such isogenic models enable subtle phenotypes to be examined, which might not be possible when comparing cells derived from two different individuals (Sandoe and Eggan, 2013). This approach is becoming a standard practice in the field.

Another powerful application of CRISPR/Cas9 technology is the introduction of exogenous sequences into a specific genomic locus *via* the HDR pathway to serve as a reporter for the activity of that gene (Hockemeyer and Jaenisch, 2016). When this approach is used to fuse a fluorescent protein sequence or an epitope sequence to an endogenous open reading frame (also called endogenous/in frame tagging), the subcellular localization and dynamics of the encoded fusion protein can be analyzed under endogenous physiological conditions (Roberts et al., 2017). In addition, the knock-in of reporter genes to label specific cell populations enables phenotypic analyses to be carried out in the cell types of interest (Nakano et al., 2012; Doyon et al., 2011). In particular, the insertion of reporter genes into developmentally important loci such as FEZF2 (Ruby and Zheng, 2009), OLIG2 (Xue et al., 2009), or NKX2.1 (Goulburn et al., 2011) has facilitated the identification and characterization of target neuronal cell types that can be used for disease modeling or transplantation. However, inserting a large sequence requires HDR with a correspondingly large, exogenously provided repair template, an inefficient process in human cells. Nevertheless, endogenous tagging offers a major improvement over conventional overexpression systems or bacterial artificial chromosome (BAC) approaches (de Dieuleveult et al., 2016), especially for live imaging (Roberts et al., 2017) and functional studies (Xiong et al., 2017; Savic et al., 2015). The ability to endogenously tag proteins allows analysis of the biochemical activity of epilepsy-related proteins for which there are no validated antibodies. Combined with phenotypic analysis in patient iPSC models, this approach provides a key molecular tool to determine underlying cellular mechanisms.

Despite the rapid evolution of genome editing, the approach still has multiple technical challenges that prevent efficient genome editing in established iPSCs (Zhang et al., 2017). First,

unwanted off-target mutations may occur. Although recent whole-genome sequencing results show that off target editing by CRISPR/Cas9 is very low in human stem cells (Smith et al., 2014; Suzuki et al., 2014; Veres et al., 2014), this problem has been further reduced through additional strategies such as designing sgRNAs with very high specificity scores, using a modified Cas9 nuclease with improved binding specificity to sgRNAs, testing sgRNAs with different lengths and PAM sequences, and the delivery of purified gRNA and Cas9 protein as a CAS9-ribonucleoprotein (RNP) complex. Second, a clonal line with homogeneous genetic background is difficult to obtain from established iPSCs that do not recover well from single-cell clonal isolation processes (Watanabe et al., 2007). The addition of a rho-associated coiled-coil kinase (ROCK) inhibitor (Watanabe et al., 2007), commercially available supplements for single cell isolation, or physiologic oxygen (2%) culture conditions (Forsyth et al., 2006) have been reported to enhance clone recovery during this harsh selection process. Some of these treatments also likely reduce the development/accumulation of spontaneous chromosomal aberrations over prolonged time in culture (Forsyth et al., 2006).

An alternative strategy to overcome the obstacle of making clonal genome-edited iPSC lines is to perform simultaneous reprogramming and CRISPR/Cas9 gene editing from somatic tissues. This approach avoids multiple rounds of clonal picking, which enables the generation of multiple edited and unedited iPSC lines simultaneously. It also requires considerably less time and resources compared to conventional multi-step protocols that can take at least several months to complete (Tidball et al., 2017; Howden et al., 2015). However, this approach is much more suitable for gene knockout than for HDR-based mutational insertion/correction, which is the less efficient of the two processes. Third, the efficiency of seamless genome editing of specific mutations or correction of patient mutations through HDR in the absence of selectable markers is very low. It can be painstakingly difficult to isolate an edited clonal line, which often makes it time- and cost-prohibitive. However, several factors may be used to improve the process. One is the delivery of purified gRNA and Cas9 protein as CAS9-ribonucleoprotein (RNP) complex to avoid the inconsistency of *in vivo* protein transcription and translation. This approach improves the efficiency of genome editing in human stem cells (Jinek et al., 2013; Kim et al., 2014; Lin et al., 2014) and reduces off-target mutations associated with plasmid transfection and indels in the allele that does not need to be modified given the reduced temporal availability of the Cas9 protein (Kim et al., 2014). Chemically or



structurally modified sgRNAs that bind to Cas9 nuclease more tightly and are less prone to degradation may also significantly enhance the efficiency of CRISPR/Cas9 mediated genome editing (Dang et al., 2015; Hendel et al., 2015). Lastly, compounds that either inhibit the NHEJ pathway (i.e. SCR7 and RS-1) or enhance HDR efficiency (i.e. L755507, brefeldin A, E1B55K and E4orf6) can improve the efficiency of mutational insertion/correction in human stem cells, although the *in vitro* toxicity of these small molecules and proteins still requires extensive evaluation (recently reviewed in Zhang et al., 2017).

More recently, hPSC lines with stable or inducible expression of Cas9 protein through lentiviral integration have been developed that allow genome-wide screening. The screening is done by delivering thousands of gRNAs (several gRNA libraries are available) into stem cells with high efficiency (Cao et al., 2016; Chen et al., 2015). For human use, it will be essential to reduce CRISPR off-target effects, improve editing efficiency, and exploit novel delivery strategies at a low cost and with high safety (not further discussed here). Nevertheless, the CRISPR/Cas9 based genome-editing system has become a powerful platform for helping investigators determine how a specific mutation affects cellular function and for generating high-throughput platforms for drug screening in a dish.

## **2-D Neural Differentiation**

Differentiating hPSCs into disease-relevant neural cell types offers great potential for studying the development and progression of neurological disorders. One of the goals of such studies involving genetic epilepsies is to generate cortical networks *in vitro* that closely resemble those found *in vivo*. Various protocols have been developed to derive neural cells from hPSCs that are most relevant to epilepsy, including cortical-like excitatory neurons (Chambers et al., 2009; Shi et al., 2012a), cortical-like inhibitory neurons ( $\gamma$ -aminobutyric acid (GABA)-expressing interneurons) (Liu et al., 2013a; Maroof et al., 2013; Nicholas et al., 2013), and hippocampal-like neurons (Yu et al., 2014; Sarkar et al., 2018). Recent developments in optimizing the protocols of neural differentiation also make it possible to generate astrocytes (Tchieu et al., 2019; Canals et al., 2018; Li et al., 2018), midbrain dopamine neurons (Kriks et al., 2011; Swistowski et al., 2010) and spinal motor neurons (Hu and Zhang, 2009; Karumbayaram et al., 2009) from hPSCs. Most of these protocols involve the

application of small molecules to hPSC monolayers or the forced expression of specific transcription factors. Small molecule differentiation can recapitulate some *in vivo* neurodevelopmental processes such as inducing ventricular zone (VZ)-like structures and transcriptional signatures. However, the resulting neural cultures are often heterogeneous and vary between cell lines (Chandrasekaran et al., 2017). Compared to small molecule differentiation, induced differentiation using transcription factors allows rapid generation of more homogeneous cultures that exhibit reproducible neuronal properties (2 weeks vs. 4-6 weeks for small molecule differentiation) (Zhang et al., 2013). However, the induced differentiation approach bypasses normal signaling pathways during neural development such that some developmental phenotypes in hPSC models of neurodevelopmental disorders may be difficult to interpret or completely absent (Schafer et al., 2019). Below, we focus on the differentiation of forebrain cortical-like excitatory and inhibitory neurons that are relevant to the epilepsies.

**Small molecule differentiation:** Based upon knowledge of the molecular cues underlying embryonic brain development, various protocols have been developed to direct hPSCs towards specific neuronal cell lineages. To differentiate iPSCs into forebrain neurons, one of two categories of protocols is typically used: dual inhibition of the SMAD signaling pathways (BMP and TGF- $\beta$ ) in a monolayer culture (Chambers et al., 2009; Shi et al., 2012b; Qi et al., 2017; Maroof et al., 2013), or embryoid body (EB) differentiation (Li and Zhang, 2006; Zhang et al., 2001; Zeng et al., 2010; Kim et al., 2010; Reinhardt et al., 2013; Liu et al., 2013a). SMAD signaling is inhibited with proteins such as Noggin and Dickkopf-1, or small molecule inhibitors, such as SB-431542, LDN193189, and dorsomorphin, to promote the differentiation of PSCs into a forebrain neural lineage. In the monolayer protocols, iPSCs are plated as an adherent monolayer followed by rapid induction of a neuroepithelial sheet through dual inhibition of the SMAD signaling pathways (Noggin and SB-431542 used in Chamber et al., 2009, SB-431542 and dorsomorphin used in Shi et al., 2012b, SB-431542 and LDN193189 used in Qi et al., 2017). In EB protocols, hPSCs are allowed to form aggregates in suspension in the absence of exogenous growth factors or small molecules. EBs are then plated onto an adherence-promoting substrate and cultured with dual SMAD inhibitors, promoting formation of definitive neuroectoderm (Zeng et al., 2010; Li and Zhang, 2006; Zhang et al., 2001). Alternatively, the EB method has been modified by the direct application of dual SMAD inhibitors into hPSC aggregate culture, and then cell aggregates are plated as adherent cultures (Reinhardt et al., 2013;

Kim et al., 2010). After definitive neuroectoderm develops and organizes into neural rosette-like structures, the neural rosettes are selected for continued growth and differentiation. Following the rosette selection, cortical development of the monolayer cultures is coupled with sequential expression of markers for the appropriate cortical laminar projection neurons.

Compared to the monolayer protocol, EB aggregate protocols generate a higher yield and purity of neurons (Muratore et al., 2014), and also result in slightly more mature neurons with longer neurites (Chandrasekaran et al., 2017). Notably, electrophysiological analysis did not reveal significant differences between these two differentiation protocols and indicated that human neurons cultured *in vitro* require prolonged culture durations to reach synaptic maturity (Chandrasekaran et al., 2017). Most protocols generate functional but still immature cortical neurons after approximately 4-6 weeks from the start of the differentiation. One recent report showed that early-born cortical neurons with functional electrophysiological properties could be generated within 8-16 days with combinatorial application of six pathway inhibitors (Qi et al., 2017), although this has not yet been replicated. Variations among the differentiation protocols can result in a bias toward development of either excitatory or inhibitory neurons, depending upon modulation of the sonic hedgehog (SHH) pathway after the development of a primitive neuroepithelium. For example, one can generate GABAergic interneurons by patterning the neuroepithelium into a homogenous population of NKX2.1-expressing MGE-like progenitors in about 6 weeks using SHH and/or the smoothed activator purmorphamine to activate the SHH pathway (Liu et al., 2013a; Maroof et al., 2013).

**Transcription factor induced differentiation:** Compared to the small molecule differentiation method, a quicker way to generate neurons from hPSCs is to force the expression of lineage-determining transcription factors. We refer here to these transcription factor-induced neurons as iNeurons. The induced expression of Neurogenin-2 (NGN2), for example, allows one to generate functional excitatory neurons within 2 weeks (Zhang et al., 2013). Analysis of these neurons shows an overall similar transcriptional expression profile except for lower expression of deep-layer neuronal markers, such as TBR1 (Pak et al., 2015), compared to that of those derived from EBs. These NGN2-induced neurons display short-term plasticity, synaptic function and modulation, and the ability to functionally integrate when transplanted into the mouse brain (Pak et al., 2015; Zhang et al., 2013). Transient expression of a combination of the transcription factors ASCL1 and DLX2, or these two

factors plus LHX6 and a specific microRNA generates functional GABAergic interneurons from hPSCs with a high degree of synaptic maturation (Sun et al., 2016a; Yang et al., 2017). These inhibitory iNeurons consist of various populations of GABAergic neurons that express subtype-specific markers and display inhibitory synaptic function. They can also integrate into host synaptic circuits after grafting *in vivo* (Sun et al., 2016a). When co-cultured with NGN-2 excitatory iNeurons, the generation of these defined populations of functionally mature human GABAergic iNeurons provides a new platform for the study of diseases affecting inhibitory synaptic transmission. A remaining challenge is that none of the current protocols reliably generates parvalbumin-expressing fast-spiking interneurons, a cell type critical for many epilepsies and other neurological and psychiatric disorders.

### **3-D Human Brain Organoid Models**

#### **Generation of brain organoids**

The human brain is one of the most complex organs in the animal kingdom, and has more types of neurons and regional diversity than that of other mammals (Geschwind and Rakic, 2013; Miller et al., 2019). One critical aspect of human brain development is the generation of an outer subventricular zone (oSVZ), which contains a large population of outer radial glia (oRG; also known as basal RG - bRG) progenitor cells. oRG are present in large numbers in gyrencephalic primates (Fietz et al., 2010; Hansen et al., 2010; Martinez-Cerdeno et al., 2012) and are considered pivotal to the evolutionary increase of human cortex surface size and complexity (Lui et al., 2011). The oRG population is largely absent during mouse brain development (Wang et al., 2011). Therefore, human cellular models provide a unique opportunity to study this cell population. Even more compelling would be to study these and other neural progenitors in the developing human brain using a cell culture system that models the 3-D architecture of human forebrain neural progenitor zones and the developing cortical mantle. A recently developed cell culture technology, known as cerebral organoids, brain organoids or cortical spheroids, now enables this approach. The general method involves growing hPSC aggregates in suspension such that they differentiate into self-organizing cerebral-like 3-D neural structures (Eiraku et al., 2008; Lancaster et al., 2013). Depending upon the specific protocol, the structures resemble diverse regions of brain and recapitulate several key *in vivo*

features of brain organogenesis. Thus, they are attractive models for studies of unique aspects of brain development, and allow modeling of human brain disorders in 3-D. A growing number of protocols have been developed to differentiate organoids that partly recapitulate specific CNS regions such as the hippocampus (Sakaguchi et al., 2015), midbrain (Jo et al., 2016; Monzel et al., 2017), hypothalamus (Qian et al., 2016), cerebellum (Muguruma et al., 2015), thalamus (Xiang et al., 2019), oligodendrocytes/white matter (Marton et al., 2019), anterior pituitary (Ozone et al., 2016) and retina (Eiraku et al., 2011). Here we highlight recent advances in generating forebrain cerebral organoids that contain cortical glutamatergic neurons and cortical interneurons.

Although neurons in monolayer cultures can develop functional synapses and form neuronal networks, traditional 2-D cultures are still limited in their ability to recapitulate human brain cytoarchitecture and to be maintained for the extended time periods necessary for human neural development. Neurons in brain organoids arguably show greater neuronal maturation than those from 2-D monolayer-derived neurons (Pasca et al., 2015). The decreased maturation potential of 2-D cell cultures may reflect a lack of the complex extracellular environment necessary to generate appropriate cell-cell connections and extracellular matrix interactions. This shortcoming and the ability for prolonged (> 1.5 years) culturing of organoids make 3-D tissue-like structures a more promising model to achieve mature human brain-like networks. The 3-D culture models are not just more heterogeneous and complex than 2-D neural cultures, but also more physiologically relevant to structures of human brain in terms of the spatial organization of tissues and electrophysiological properties of neural cells (Wang, 2018; Amin and Pasca, 2018; Chen et al., 2019b; Di Lullo and Kriegstein, 2017; Kelava and Lancaster, 2016; Kretzschmar and Clevers, 2016). Cerebral organoids generate a laminar organization that partly recapitulates human cortical development, such as defined multi-layer progenitor zone organization (VZ and inner/outer SVZ) and diverse neural cell types with identities of potentially all six layers of developing neocortex. Many studies have described the appearance of astrocytes (Dezonne et al., 2017; Pasca et al., 2015; Qian et al., 2016; Sloan et al., 2017) and less frequently oligodendrocytes (Matsui et al., 2018) in cortical organoids during later stages of differentiation. More recently, human oligodendrocyte-lineage containing brain organoids were developed to generate oligodendrocytes that are transcriptionally similar to primary

oligodendrocytes and mature both morphologically and electrophysiologically over time (Marton et al., 2019; Kim et al., 2019; Madhavan et al., 2018).

Protocols for generating cerebral organoids from hPSCs are evolving rapidly in this fast moving field. These protocols often require two processes: the establishment of neural identity and the recapitulation of 3-D structural organization. One approach for generating cerebral organoids is known as the “intrinsic protocol” based on the self-organizing potential of stem cells. As a result, each organoid is typically comprised of diverse cell types found in forebrain, hindbrain, and retina (Lancaster and Knoblich, 2014a; Lancaster et al., 2013). More recently, this self-organizing approach has been modified to generate region-specific brain organoids. The method depends on the specific temporal application of extrinsic signaling molecules (reviewed in Di Lullo et al., 2017) that pattern EBs and neural progenitors to adopt the fate of the desired brain region (Birey et al., 2017; Pasca et al., 2015; Qian et al., 2016; Xiang et al., 2019; Xiang et al., 2017). For generating forebrain cortical organoids, hPSCs are allowed to develop into EB-like aggregates, which have 3 germ layer differentiation potential. Subsequent suspension culture of EBs in a minimal medium, in the presence of SMAD signaling inhibitors, inhibits mesoderm and endoderm formation and instead promotes the formation of neural rosettes, a polarized organization of neuroepithelial cells and the later source of diverse neural progenitors. EBs are then embedded in a Matrigel scaffold (Lancaster et al., 2013; Qian et al., 2016; Quadrato et al., 2017) or cultured in medium with a low concentration of Matrigel (Kadoshima et al., 2013; Watanabe et al., 2017; Velasco et al., 2019) to support the self-organization of the neuroepithelium and to induce the correct polarity signals for the development of an apicobasally polarized neuroepithelial bud. The neuroepithelium further develops into structures that are remarkably similar to the VZ and SVZ regions of the developing human brain. These include an oSVZ region that contains oRG cells that are crucial for human cortical expansion (Miller et al., 2019). The support by Matrigel in some protocols seems to be critical for the building of *in vitro* brain structures (Qian et al., 2016). Later agitation, such as those provided by spinning bioreactors (Qian et al., 2016), or mechanically cutting the organoids (Watanabe et al., 2017) to remove the necrotic center promotes the development of much larger and more uniform cortical organoids. The organoids contain fluid-filled cavities that resemble brain ventricles (Lancaster et al., 2013), and generate neurons with identities of deep and superficial layers of neocortex. A simplified approach for cortex-

specific organoid generation that does not require extracellular matrix support and media agitation also yields cortical spheroids with well-defined segregation of superficial and deep cortical layers (Pasca et al., 2015; Xiang et al., 2019; Xiang et al., 2017).

Much of the initial effort was focused on cortical organoids, emphasizing the development of glutamatergic cortical neurons. Few interneurons are found in standard cerebral organoids/cortical spheroids (Pasca et al., 2015; Qian et al., 2016) because they are generated from protocols that promote dorsal cell fates through inhibition of WNT and SHH pathways and thus lack tissue resembling the ventral ganglionic eminences. To rectify this problem, ventralized organoids containing GABAergic interneurons have been generated. Similar to the generation of GABA interneurons in 2-D culture, the EBs can be patterned to ventral forebrain organoids/spheroids by combined activation of the SHH pathway and WNT inhibition (Bagley et al., 2017; Birey et al., 2017; Lancaster and Knoblich, 2014a; Xiang et al., 2017), or SHH pathway activation alone (Watanabe et al., 2017). These ventral organoids develop ventricular zone (VZ)-like structures expressing *NKX2-1* and later generate GABAergic interneuron subtypes. They also can be fused to dorsalized glutamatergic organoids *in vitro* (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017). The fused forebrain assemblies demonstrate the migration of interneurons from the ventralized (GE-like) organoid into the dorsalized organoid and establishment of circuits with glutamatergic cells, mirroring normal developmental processes observed in the fetal forebrain (Birey et al., 2017; Xiang et al., 2017; Bagley et al., 2017).

In terms of neuronal function, organoids can be grown for more extended periods of time compared to 2-D cultures, allowing for increased neuronal maturation. Initial studies showed the presence of slow neuronal calcium waves and action potentials induced by current injections (Lancaster et al., 2013; Pasca et al., 2015; Qian et al., 2016). Both glutamatergic and GABAergic synaptic transmission is present in cortical organoids (Qian et al., 2016). Evoked action potential firing rates and excitatory synaptic inputs increase substantially in fused forebrain organoids that are comprised of glutamatergic and GABAergic neurons in a more physiological ratio (Birey et al., 2017). Prolonged culture (up to 20 months) of human cortical spheroids also promotes astrocytic maturation (Sloan et al., 2017). Recent studies showed that spontaneous neuronal network activity could be detected in 8-month-old brain organoids through extracellular recordings with high-density silicon

microelectrodes (Quadrato et al., 2017). Loss of this activity in the presence of neurotransmitter receptor antagonists suggests the presence of synaptic networks in the organoids. Thus, this technology offers new opportunities to study both neuronal dynamics in developing human brain-like structures and the neural circuitry underlying disease mechanisms of epilepsy and other neurological disorders.

### **Assays for Measuring hPSC-Derived Neuronal Activity and Differentiation**

Various methods can be used to determine the function of neurons generated from hPSCs. Traditional electrophysiologic techniques, such as patch-clamp recordings, provide insight into intrinsic cellular and synaptic properties that may lead to epilepsy (Liu et al., 2013b; Shcheglovitov et al., 2013). Such studies can be performed on 2-D neuronal cultures, but they also can be applied to acute slices or dissociated cells from organoid cultures. Moreover, electrophysiological activity of hPSC-derived neurons transplanted into the rodent brain may be assayed in *ex vivo* brain slices (Espuny-Camacho et al., 2013; Oki et al., 2012). However, patch clamp techniques are labor intensive, and typically only permit recording from individual cells at a single time point during development. As an alternative, tissue culture plates with embedded multielectrode arrays (MEAs) may be used to measure extracellular field potentials of neuronal cultures (Mack et al., 2014; Obien et al., 2014; Pine, 1980). MEAs allow for longitudinal real-time measurements of spontaneous activity from dozens of neurons under normal culture conditions. This technique also allows for repeated recordings from different cell types grown under various experimental conditions in multiple-well format. MEA recordings are often combined with drug application. For example, one study used such recordings to identify a drug that blocks hyperexcitability in ALS patient iPSC-derived motor neurons (Wainger et al., 2014).

Another approach for assaying functional activity of iPSC-derived neurons is to use optical  $\text{Ca}^{2+}$  imaging to detect transient increases of calcium concentration in active neurons. This imaging is often accomplished by using either bulk loading of chemical-based fluorescent calcium indicators (Krey et al., 2013), or by expressing genetically encoded indicators of  $\text{Ca}^{2+}$  concentration such as GCaMP6 (Grienberger and Konnerth, 2012; Hamel et al., 2015; Peron et al., 2015). For example, in studying Timothy syndrome caused by a mutation in the  $\text{Ca}_v1.2$  calcium channel, the investigators



used the Fura-2 dye to show that the phenotype of dendrite retraction is independent from calcium flux through the channel (Krey et al., 2013). Calcium oscillations can be monitored to show network formation through automated and non-invasive imaging in 96-/384-well plates (Tortoriello et al., 2014; Collier et al., 2017; Rana et al., 2017; Jin et al., 2018). Calcium imaging has been performed in either intact or dissociated cerebral organoids to demonstrate that spontaneous surges of intracellular calcium correspond to electrical activity (Lancaster et al., 2013; Pasca et al., 2015; Xiang et al., 2017). In addition, *in vivo* two-photo calcium imaging systems can be applied for longitudinally measuring neuronal network activities of grafted organoids in rodent brain (Mansour et al., 2018b).

Although  $\text{Ca}^{2+}$  imaging is applied to multiple cells simultaneously, it typically lacks the temporal resolution to reliably distinguish individual action potentials and does not reliably measure local field potentials. In contrast, high-density silicon microelectrodes have recently been developed to obtain scalable spatially oversampled neural recordings in live mammalian brain. Each probe comprises up to 1000 electrode pads on 5 shanks with 200 recording sites per shank (Scholvin et al., 2016). This approach makes it possible to record large populations of single unit activity from multiple brain structures in freely moving animals. Application of this technology to 8-month-old human brain organoids enabled the detection of spontaneously active neuronal networks (Quadrato et al., 2017).

In addition to electrophysiologic and calcium imaging studies, image-based techniques are available to study the morphology of iPSC-derived neurons. Measuring neurite outgrowth in culture is perhaps the most common phenotypic assay to study neuronal maturation *in vitro* (Yeyeodu et al., 2010). In addition to neurite length, the complexity of neurite branching, number of extensions per cell, and number of neurites per cell can be measured by image segmentation. Sholl analysis (Scholl, 1954) is often used and provides the number of dendrite intersections against the radial distance from the soma center (Binley et al., 2014). Recent developments in automated live-cell imaging systems allow for longitudinal and high-throughput phenotypic screens of neurite outgrowth of iPSC-derived neurons in monolayer cultures (Camarena et al., 2014; Oudin et al., 2011; Bamba et al., 2017; Cavaliere et al., 2017). Assessing neuronal morphological changes is helpful for understanding the mechanisms of neurological disorders, as well as quantifying the effects of potential drug treatments (Sherman and Bang, 2018).

Lastly, molecular characterization such as transcriptome analysis offers a powerful strategy to provide valuable insight into complex biological systems, including the CNS, using molecular signatures. Conventional bulk transcriptome analysis using RNA sequencing (bulk RNA-seq) provides the average transcriptional patterns for entire cell populations. In contrast, single-cell RNA sequencing (scRNA-seq) enables high-throughput (up to thousands of cells per experiment) analysis of an entire transcriptome at a single-cell level (Brazovskaja et al., 2019; Hwang et al., 2018; Picelli, 2017). scRNA-seq often involves four major steps: isolating single cells, capturing their transcripts, generating sequencing libraries in which the transcripts are mapped to individual cells, and analyzing and interpreting scRNA-seq data. Due to technical noise (i.e. low amount of starting material and high drop out rate) and inherent biological variation, scRNA-seq often produces noisier and more variable data than bulk RNA-seq (Chen et al., 2019a). In bulk RNA-seq experiments, batch effects can be minimized by preparing sequencing libraries from biological replicates in parallel and then sequencing them simultaneously. However, in scRNA-seq experiments, this is not feasible, as cells from different conditions are often captured and prepared independently due to the lower throughput of this platform (Olsen and Baryawno, 2018). Appropriate controls and a minimum of three biological replicates per condition are recommended, and also functional validation is an important step to assess the relevance of these data to the biological context (Olsen and Baryawno, 2018).

Although plenty of improvements are in need, scRNA-seq is best suited to efficiently reveal the complex cellular diversity in brain organoids (Camp et al., 2015), track the trajectories of distinct cell lineages in development (Birey et al., 2017; Xiang et al., 2017; Quadrato et al., 2017), and to understand organoid-to-organoid variability (Quadrato et al., 2017; Velasco et al., 2019). Camp and colleagues were the first to directly compare cerebral organoids with human early fetal neocortex using scRNA-seq based on the Fluidigm system and found that *in vivo* cell lineages and gene expression programs were largely recapitulated in the organoid cortical regions (Camp et al., 2015). A major limitation of this study was the low throughput of scRNA-seq technology (less than 1000 cells per experiment). Quadrato et al. substantially advanced the characterization of self-patterned whole-brain organoids using a higher throughput scRNA-seq microdroplet-based microfluidics platform (Quadrato et al., 2017). Using this Drop-seq based method the authors sequenced over 80,000 cells from 31 brain organoids and found that neurons in multiple lineages progressively matured over time

at the transcriptome level. Notably, the authors also reported significant organoid-to-organoid variability related to bioreactor-based batch effects identified in the scRNA-seq analysis (Quadrato et al., 2017), which was confirmed by a recent study using a similar approach to generate self-patterned multi-region brain organoids (Velasco et al., 2019).

### **iPSC Models of Genetic Epilepsies**

iPSC models of genetic epilepsies include those in which epilepsy is the defining feature/symptom, such as DS or STXBP1-Related Epileptic Encephalopathy, and disorders in which epilepsy is a variable feature associated with other neurodevelopmental abnormalities, including TSC, Rett syndrome, Fragile X syndrome and Timothy syndrome. Many of the recent studies of epilepsy-associated genetic diseases have focused on those with an onset in infancy or early childhood given the relative ease of modeling defects in early neural development with hPSCs. Although an epileptic-like phenotype is not as well-defined using *in vitro* models as it is in animal models, *in vitro* models have been used to show increased spontaneous action potential firing rates and increased bursting and synchrony as correlates of epileptic-like activity (DeLorenzo et al., 1998). Patient-derived iPSC models for many of these diseases have also demonstrated altered neuronal morphology including soma size, neurite outgrowth, synapse formation, and dendritic spine length (Costa et al., 2016; Patzke and Sudhof, 2016; Yamashita et al., 2016). 2-D iPSC models have been particularly important for analyzing parameters such as gene expression, cell morphology, neuronal excitability, and synapse formation since 2-D cultures are easy to manipulate and readily available (Flaherty and Brennand, 2017; Wen et al., 2016; Soliman et al., 2017). Several reviews have summarized the modeling of genetic epilepsies and other related neurological disorders in monolayer neurons (Parent and Anderson, 2015; Tidball and Parent, 2016). However, 3-D brain organoid modeling, with its more complex laminar organization, cell diversity, and mature neural networks, opens the door to new possibilities for modeling epilepsy related disorders. Benefits and disadvantages of different model systems for human brain disorders have been summarized recently (Chen et al., 2019b). In subsequent sections of this review, we describe using 2-D and/or 3-D hPSC-derived cultures to model the abovementioned genetic epilepsies and related neurodevelopmental disorders with a focus on human brain-specific phenotypes.

**Dravet syndrome (DS)** is a severe childhood epileptic encephalopathy that typically presents in infancy with treatment-resistant, prolonged seizures, and subsequent developmental delay and intellectual disability (Dravet et al., 2005). In over 80% of cases, the cause is a de novo heterozygous mutation in the SCN1A gene, which encodes the voltage-gated sodium channel (VGSC) Na<sub>v</sub>1.1, leading to haploinsufficiency (Meisler and Kearney, 2005). Opening of VGSCs results in an influx of sodium ions necessary for the firing of action potentials, making haploinsufficiency of this sodium channel seem unlikely to cause epilepsy. Prior heterozygous knockout or human mutant SCN1A knock-in mouse models suggested an answer to this apparent paradox with the finding of selective decreases in interneuron excitability predicted to cause disinhibition and cortical hyperexcitability (Yu et al., 2006; Ogiwara et al., 2007). However, recent work with DS patient iPSCs and additional mouse studies support a more complex pathophysiology.

Several groups generated iPSCs from DS patients, differentiated them into excitatory and inhibitory neurons and used whole-cell patch-clamp recordings to examine neuronal electrophysiology. One group unexpectedly showed increased (rather than the expected decrease seen in mouse interneurons) sodium current ( $I_{Na}$ ) density and excitability in both presumptive excitatory and inhibitory neurons derived from 2 DS subjects compared to unrelated controls (Liu et al., 2013b). This result was supported by another study of one DS patient that also found increased  $I_{Na}$  in excitatory neurons (Schafer et al., 2019). However, several other groups studies have reported decreased  $I_{Na}$  in iPSC-derived interneurons with mutant SCN1A-linked DS (Kadoshima et al., 2013; Liu et al., 2017; Sun et al., 2016b), and one of these studies used a more advanced protocol to generate interneurons of a medial ganglionic eminence-like origin and also showed unchanged  $I_{Na}$  in excitatory neurons (Sun et al., 2016b). The variability in results may be secondary to differences in the methods of neuronal differentiation, neuronal subtypes, or the age and maturity of the neurons. Subsequent DS mouse model studies complicated the picture further, showing a developmental transition where initially interneurons have decreased  $I_{Na}$  and hypoexcitability, but then, due to unknown compensatory mechanisms, the interneurons normalize and excitatory neurons show increased  $I_{Na}$  and excitability (Favero et al., 2018; Mistry et al., 2014). Taken together, the DS human iPSC and mouse data suggest that interneuron dysfunction initiates the epileptic encephalopathy, but resultant abnormalities in excitatory neurons may maintain the epileptic state.

**STXBP1-Related Epileptic Encephalopathy** is a severe epilepsy that typically begins in early infancy and results from *STXBP1* haploinsufficiency (Saitou et al., 2008). *STXBP1* is a protein that is critical for effective presynaptic vesicle release. Several groups have found abnormal phenotypes in both patient-derived iPSC and genetically modified conditional hESC models of *STXBP1*-Related Epileptic Encephalopathy. The abnormalities include decreased *SYNTAXIN1* expression/altered localization, decreased neurite outgrowth and reduced neurotransmitter release associated with heterozygous *STXBP1* loss of function (Patzke and Sudhof, 2016; Yamashita et al., 2016). In homozygous *STXBP1* null hESC lines, severe neurodegeneration also occurs (Patzke and Sudhof, 2016).

**TSC** is a multisystem disorder that most prominently affects the skin, brain, lungs, kidneys and heart. The disorder is caused by autosomal dominant de novo or inherited mutations in either the *TSC1* or *TSC2* gene that encodes hamartin or tuberlin, respectively (Au et al., 2007). The mutations are loss of function and they lead to variable hamartomas in these tissues that reflect abnormal cell growth. The variability is thought to be due to the requirement for “second hit” mutations leading to loss of heterozygosity and subsequent hamartomas. *TSC1* and *TSC2* form part of a heterotrimeric complex that represses the mechanistic target of rapamycin (mTOR) pathway, and loss of function results in mTOR hyperactivity that causes aberrant cell growth (Hasbani and Crino, 2018). In the brain, the main abnormal growths are cortical tubers, a type of focal cortical dysplasia (FCD), that most commonly present as seizures at any point in life from infancy to adulthood. Developmental delay, psychiatric disturbances, intellectual disability and autism spectrum disorder are also often seen. Less frequently, subependymal giant cell astrocytomas arise in the brain and may lead to hydrocephalus.

Human PSC models of TSC have recently been developed to complement previous animal studies (see (Blair and Bateup, 2019)). Costa and colleagues (Costa et al., 2016) generated heterozygous and homozygous *TSC2* knockout hESC lines via genome editing and found disease-relevant phenotypes, predominantly in homozygous lines. The phenotypes included increased neural progenitor proliferation, delayed neuronal differentiation, enhanced generation of astrocytes, and increased neuronal soma size and dendritic complexity. In terms of functional effects, *TSC2* knockout hESC-derived (mainly excitatory) neurons showed reduced evoked action potential firing and

decreased spontaneous and miniature excitatory postsynaptic currents, findings that do not seem to explain the common epilepsy phenotypes in patients, although the function of cortical inhibitory-like neurons was not specifically assessed. The morphological and functional phenotypes were associated with increases in markers of mTOR pathway hyperactivity, again to a much greater degree in homozygous *TSC2* null cells, and they were reversed by the mTOR inhibitor rapamycin. Multiple groups subsequently found more subtle but similar phenotypes in haploinsufficient *TSC1* or *TSC2* patient iPSC-derived forebrain neurons or purkinje cells (Li et al., 2017a; Zucco et al., 2018; Nadadhur et al., 2019; Sundberg et al., 2018).

Recently, Blair and colleagues combined brain organoid and CRISPR gene editing methods to model TSC in 3-D cultures (Blair et al., 2018). They generated hESC lines with either constitutive heterozygous or homozygous null *TSC1* or *TSC2* alleles, or lines with 1 null and 1 conditional *TSC2* allele, the latter to model a second hit during various stages of brain organoid development after the viral introduction of Cre to delete the second *TSC2* allele, while also activating a Cre reporter placed in a safe harbor locus. Similar to previous 2-D findings in gene edited TSC lines (Costa et al., 2016), phenotypes were present in cortical spheroids generated from homozygous null lines and largely absent in those derived from heterozygous lines. The changes included expression of markers indicating increased mTOR pathway activation, decreased neuronal and increased astrocytic differentiation, and enlarged, dysmorphic neurons and glia resembling TSC patient cortical tubers. Remarkably, focal Cre delivery to delete the conditional second allele in brain organoids from lines with constitutive haploinsufficiency led to the focal generation of tuber- or FCD-like abnormalities in the organoids. The authors replicated the findings by similarly inducing a focal knockout of a conditional allele generated from TSC patient iPSC-derived spheroids as a second hit. They also showed that early rapamycin treatment of the brain organoids prevented development of the abnormal phenotypes. Given the lack of findings in heterozygous TSC loss-of-function organoids, this elegant work provides strong support for the two-hit hypothesis of TSC pathophysiology.

**Rett syndrome (RTT)** is arguably the most studied epileptic disorder using iPSCs. RTT is an X-linked neurodevelopmental disorder caused primarily by mutations in *MECP2*, which encodes a multifunctional epigenetic regulator (Ip et al., 2018). Strikingly, 50%–90% of RTT patients have

seizures (Dolce et al., 2013). iPSC models of RTT patients from several groups showed that RTT mutant neurons display a decrease in neuronal soma size, neurite outgrowth, synapse formation, and spontaneous activity, compared to wild-type MECP2-expressing neurons derived from isogenic RTT patient iPSC lines (Cheung et al., 2011; Marchetto et al., 2010). Marchetto and colleagues also demonstrated rescue of the *MECP2* mutation associated phenotypes via insulin-like growth factor-1 (IGF1) treatment (Marchetto et al., 2010). Interestingly, mutant RTT astrocytes derived from RTT patients have adverse effects on the morphology and function of wild-type iPSC-derived neurons (Williams et al., 2014). This finding demonstrates the critical roles played by glia in RTT pathology and the need to consider astrocytic contributions to epileptogenesis. Although postnatal functions of MECP2 have been extensively investigated, the role of MECP2 in early brain development remains poorly understood. Mellios and colleagues used RTT patient-derived iPSCs and MECP2 knockdown by shRNA to identify novel MECP2-targeted miRNAs during neuronal development. They also used MECP2-deficient and patient-derived monolayer culture and cerebral organoids to identify defects in neurogenesis, neuronal differentiation and migration (Mellios et al., 2018). They found a novel signaling pathway mediated by MECP2-targeted miRNAs that might influence neurogenesis and early brain development (Mellios et al., 2018). This study also suggests that early disruptions in brain development could result in increased susceptibility to subtle postnatal deleterious effects on brain maturation and plasticity, thus contributing to the full pathogenesis of neurodevelopmental disorders.

**Miller-Dieker syndrome (MDS)** is a severe congenital form of lissencephaly. It is caused by a heterozygous deletion of chromosome 17p13.3, which leads to a severe malformation of cortical development (Matarese and Renaud, 2009; Nagamani et al., 2009). Affected children suffer from feeding difficulties, severe intellectual disability, developmental delay, and seizures. Past studies of this disease largely relied on mouse models, which have the obvious disadvantage of being naturally lissencephalic. Although human brain organoids have not yet recapitulated gyrification, they contain most relevant cell types and structures likely to be involved in disease development. Patient iPSC-derived human brain organoids were recently used in two studies to investigate how MDS affects human progenitor subtypes (such as oRG cells) that control neuronal output and influence brain topology. One study showed a neuronal migration defect and severe apoptosis of the founder neuroepithelial stem cells in the VZ-like region. The authors also found a migration defect in the oRG

cells, a cell type that is largely absent from lissencephalic rodents but critical for human neocortical expansion (Bershteyn et al., 2017). Similarly, Iefremova and colleagues used patient-specific forebrain organoids to investigate pathological changes associated with MDS. They found that patient-derived organoids are reduced in size, a change accompanied by a switch from symmetric to asymmetric cell division of VZ radial glial cells (Iefremova et al., 2017). In addition, they identified non-cell-autonomous defects in the WNT signaling pathway. These important studies provide insight into the cellular pathogenesis of lissencephaly and demonstrate the utility of using brain organoids to model human-specific phenotypes.

**PTEN macrocephaly:** The phosphatase and tensin homolog (PTEN)-protein kinase B (PKB/AKT) cascade regulates human cortical development (Lee et al., 2012; Mirzaa et al., 2013), and PTEN heterozygous loss-of-function mutations have been found in patients with macrocephaly (Butler et al., 2005; Marchese et al., 2014). In the human cerebral organoid system, deletion of the *PTEN* gene by CRISPR/Cas9-mediated genome editing increases AKT activity in human neural progenitors, promotes cell cycle re-entry, and transiently delays neuronal differentiation (Li et al., 2017b). These alterations result in a pronounced expansion of the radial glia and intermediate progenitor cells. In this study, both mouse and human brain organoids were used to model macrocephaly and showed that the lack of PTEN led to a significant increase in size (recapitulating macrocephaly), but only knockout human organoids displayed substantially increased surface folding. The phenotypic differences might reflect inherent species differences in signaling regulation, cellular response, or brain cytoarchitecture. The use of human brain organoids thus can produce human-specific phenotypes and provide unique insights into underlying disease mechanisms.

**ZIKV infection:** Although not a genetic cause of epilepsy, congenital ZIKV virus infection is known to cause microcephaly and severe brain abnormalities in infants (Li et al., 2016; Mlakar et al., 2016; Driggers et al., 2016), and infected subjects also have a high risk of epilepsy (Pastula et al., 2017). Recent studies using human brain organoids to model ZIKV infection have demonstrated that ZIKV infection induces the death of neural progenitor cells and impairs organoid formation, providing certain mechanistic insights into disease pathogenesis (Cugola et al., 2016; Garcez et al., 2016; Qian et al., 2016). However, none of these studies recapitulated the key developmental features of the human cortex such as surface area and folding. In contrast, genetically modified PTEN mutant brain



organoids show increased surface area and folding, and were used to model the destructive effect of ZIKV infection on these key features of the human cortex *in vitro* (Li et al., 2017b). In this study, ZIKV infection led to the widespread initiation of apoptosis at the onset of surface folding and later severely impaired organoid growth both in size and surface folding. Furthermore, recent work has exploited brain organoid technology in developing drugs or screening therapeutic compounds that can mitigate the destructive actions of ZIKV infection on fetal brain (Watanabe et al., 2017; Xu et al., 2016; Zhou et al., 2017). Importantly, these findings further illustrate the strength of the human brain organoid system in modeling complex structural malformations of the developing human brain, and in recapitulating human neural progenitor-specific pathology.

### **Limitations, Challenges and Future Directions**

While the use of hPSC-based models offers many advantages, modeling epilepsy and other neurological disorders with hPSCs also presents several challenges. One issue, mentioned earlier, concerns the variability of iPSC lines within and between subjects. Using isogenic controls generated by gene editing along with multiple lines per condition partially alleviates this problem. Another critical question is how closely hPSC-derived neurons reflect those that are affected in human epilepsies. Because hPSC-derived neurons are differentiated *in vitro* for only weeks to months, they typically represent an earlier developmental stage compared to *in vivo* development. If the onset of seizures is associated with a gene expressed at a later stage of brain development, hPSC derived neurons may not reflect the critical gene alteration in patient brains. Secondly, although transcriptional profiles of 2-D hPSC monolayer neuronal cultures show remarkable preservation of *in vivo* neurodevelopmental signatures from embryonic to early fetal corticogenesis, these neurons do not mature beyond fetal stages *in vitro* (Stein et al., 2014). Different compounds or conditions have been used to accelerate the rate of maturation, including gamma secretase inhibitors (DAPT and compound E) that block notch signaling, neurotrophic factors, and co-culture with either human or rodent astrocytes (Tang et al., 2013). The lack of maturation may also be alleviated partially by using improved culture media, such as BrainPhys (Bardy et al., 2015), and with the development of mature neuronal reporters that label purely mature neurons for electrophysiological study. Moreover, these approaches may be

combined with NGN2-directed differentiation, yielding a more homogeneous population of mature neurons (Zhang et al., 2013).

A major limitation of organoid models is the relative immaturity of neuronal circuitry compared to their *in vivo* counterpart. Transcriptional profiles of cortical organoids that are cultured up to 100 days *in vitro* recapitulate those of about 16–24 week postconception human brain (Pasca et al., 2015; Camp et al., 2015). More recent studies suggest that the prolonged culture of human brain organoids leads to further maturation beyond early developmental stages (Quadrato et al., 2017; Giandomenico et al., 2019). One study found spontaneous action potentials and defined synaptic junctions in 8-month old brain organoids, but not in younger ones, suggesting that long-term culturing can promote functional neuronal connectivity (Quadrato et al., 2017). Human cortical circuits require the assembly of both glutamatergic neurons that are generated in the dorsal forebrain and GABAergic interneurons that are produced in the ventral forebrain. In the dorsal-ventral fused organoids described earlier, interneurons that migrate into cortical spheroids fired action potentials at twice the rate of cells in unfused ventral spheroids (Birey et al., 2017). This finding suggests that pre-patterned organoids can be fused to promote the formation of functional synapses and neuronal maturation. Grafting of cerebral organoids into rodent brain *in vivo* also yielded progressive differentiation and maturation (Mansour et al., 2018b). Another recent approach used to enhance maturation and decrease cell death within brain organoids involves culturing them at a liquid-air interface, which improves neuronal survival and morphology with extensive axon outgrowth following long-term *in vitro* culture (Giandomenico et al., 2019). However, many human cortical circuits develop postnatally, and may take years to mature *in vivo*. In particular, the development of parvalbumin fast-spiking interneurons in 2-D or 3-D hPSC-derived neuronal cultures has been challenging. Thus, significant challenges of circuit maturation in brain organoids remain.

Another obstacle to the application of hPSCs for disease modeling involves their limited ability to reproducibly recapitulate the diversity of neural cell types and regional specificity found in the human brain. There are numerous neuronal subtypes in human brain, organized by brain region, layer, connectivity, neurotransmitter content, and transcriptional profiles. These various neuronal subtypes make diverse contributions to the epileptic phenotype. In addition, although neurons in 3-D brain organoids have more complex anatomic connectivity and neuronal networks than 2-D

monolayer neurons do, many non-neuronal cell types found in the brain such as oligodendrocytes, meningeal cells, microglia and brain microvascular endothelial cells remain challenging to produce or incorporate into brain organoid cultures *in vitro* (Lancaster and Knoblich, 2014a; Pasca et al., 2015; Lancaster and Knoblich, 2014b). This is largely due to the lack of surrounding supportive tissues and endogenous developmental and patterning cues in the organoids. The "intrinsic" organoids mainly depend on the ability of stem cells to spontaneously acquire neural identity and to self-organize into brain-like structures. This promotes the generation of somewhat diverse brain-like regions and cell populations (Lancaster and Knoblich, 2014a). Although such diversity is appealing, it often leads to inconsistent outcome and batch effects that may conceal critical phenotypes. Thus, reproducibility and consistency are substantial challenges in these self-organizing brain organoid protocols (Brazovskaja et al., 2019)

On the other hand, recent "extrinsic" protocols create brain-region specific organoids whereby patterning is restricted by externally added morphogens and fate-specifying, and these have shown promise in improving reproducibility. For example, the dorsal forebrain organoids that were recently developed by Velasco et al., reliably generate a rich diversity of cell types similar to those in the human cerebral cortex and show consistent reproducibility in the cell types produced in these region-specific organoids (Velasco et al., 2019). Although these methods restrict the ability to generate complex brain structures compared to the "intrinsic" protocols, this shortcoming can be partially overcome by assembling different types of organoid-derived structures *in vitro*, generating so-called "assembloids". For example, fusion of human cortical organoids and human subpallial organoids creates functional human forebrain structures that contain both glutamatergic excitatory neurons and GABAergic inhibitory interneurons (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017). A similar assembloid approach has been used to reconstruct thalamo-cortical circuitry from hPSCs (Xiang et al., 2019). These approaches are especially significant for investigating epileptic-like phenotypes of interconnections between excitatory and inhibitory neurons, or those involving cortical-thalamic/thalamo-cortical networks. Tissue engineering and biomaterials will be also important for adding structural features that are normally present in human brain into organoids. For example, one group has combined hPSC-derived neural progenitor cells, endothelial cells, mesenchymal stem cells, and microglia/macrophage precursors on chemically defined polyethylene glycol hydrogels to

enable these cells to develop into 3-D neural structures and to model cellular interactions within the developing brain (Schwartz et al., 2015). Although still preliminary, this work supports the feasibility of integrating non-neural cells or tissues into brain organoids to investigate more complicated cellular interactions in the developing brain. This approach is especially important for investigations of neurodevelopmental disorders, including epilepsies, that require these cellular interactions during developmental processes such as neuronal migration, synaptogenesis, maturation and network homeostasis.

With increasing knowledge of human cortical development and progress in the development of differentiation protocols, there have been a growing number of methods that researchers can use to interrogate cellular properties of interest in brain organoids. Although there is not yet a standard practice in this field, the sampling of multiple organoids per experiment at multiple developmental time points, ideally also with organoids derived from multiple iPSC lines, is optimal to mitigate outlier effects. It will be also important for the disease features to be replicated by multiple laboratories in order to support their true relevance. While forming a "standard" protocol is unrealistic, detailed reporting of differentiation methods in published studies is an imperative practice (see Table 1 for examples of the numbers of organoids and/or cell lines analyzed in various selected studies).

## **Conclusions**

The need to create alternative models of human epilepsy has been highlighted by the difficulties/failures to translate findings from animal models to clinical therapies. Although there are still many challenges ahead, hPSC technology provides the unprecedented ability to model genetic epilepsies by growing a large number of renewable human cells in a dish and differentiating them into disease-relevant neural cell types. In particular, these models are well suited to investigate disease mechanisms and for drug screening to discover new precision therapies to treat many severe genetic epilepsies and related disorders. The approaches are facilitated by the relative ease of differentiating hPSCs to brain tissues that partially recapitulate early human neurodevelopment *in vitro*. A variety of hPSC-based models have already provided insight into mechanisms for several of the genetic epilepsies. Advances in the development of hPSC models will arise from improvements in methods to derive specific neuronal subtypes and neural circuitry in 2-D and 3-D cultures. Progress in the

development of efficient and reliable genome editing methods for hPSC lines will no doubt play a role in these advances. Lastly, improved functional assessment of neural activity and exciting higher throughput drug screening capabilities are on the horizon. Given the rapid advances already realized, hPSC-derived models hold strong promise for providing a better understanding of human brain development, disease mechanisms and new epilepsy therapies by bridging the gap between model systems and patients.

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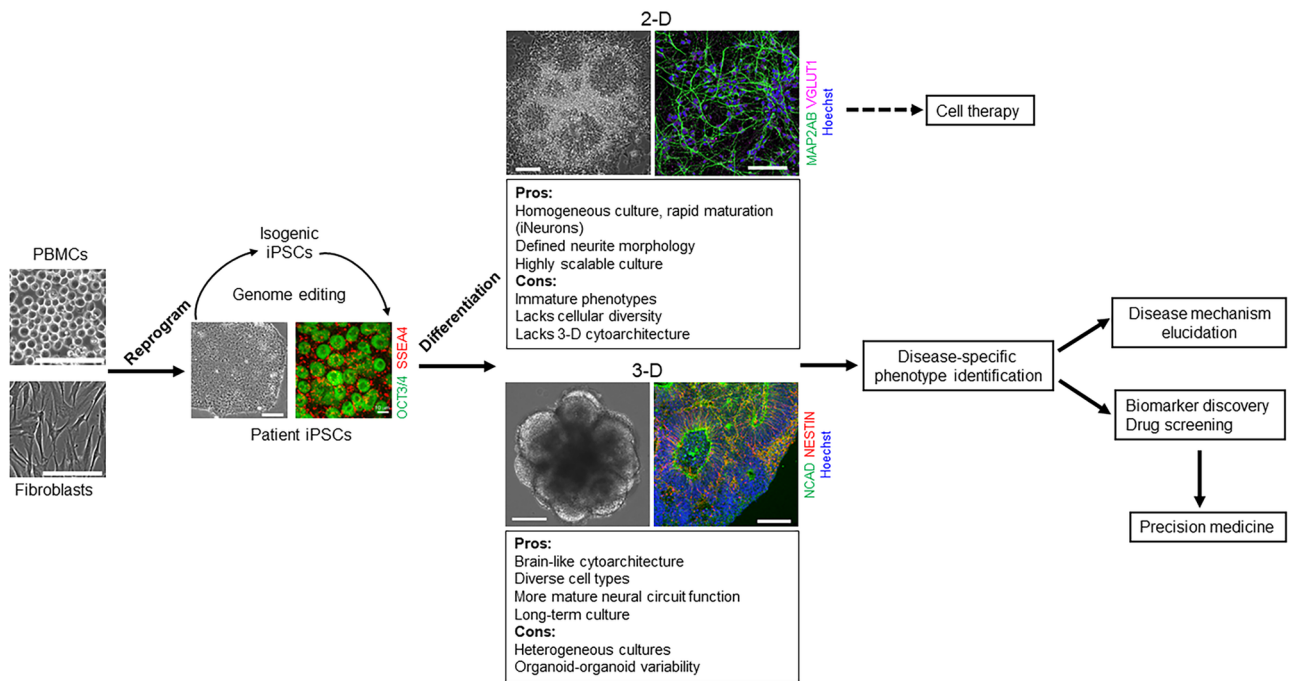
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**Figure 1:** Overview of schema for studying genetic neurodevelopmental disorders using iPSC-derived 2-D and 3-D models. Patient somatic cells, such as peripheral blood mononuclear cells (PBMCs) or fibroblasts, are reprogrammed into iPSCs and then may be differentiated into 2-D neuronal cultures or 3-D brain organoids. iPSC colonies express pluripotency-associated genes such as octamer-binding transcription factor 3/4 (OCT3/4; green) and stage-specific embryonic antigen-4 (SSEA4; red). In hPSC-derived 2-D neural cultures generated from a dual-smad differentiation protocol (Shi et al., 2012), neural VZ-like rosette structures (left) and cortical-like excitatory neurons (right) that express microtubule-associated protein 2AB (MAP2AB; green), and vesicular glutamate transporter-1 (VGLUT1; magenta) form after approximately 3 or 6 weeks of differentiation, respectively. In 3-D cortical organoid culture generated using a published protocol (by Qian et al., 2016), apicobasally polarized neuroepithelial buds (left) appear after 10 days of differentiation. Defined VZ-like structures with N-cadherin (NCAD; green) at the apical surface and neural progenitor cells immunolabeled for nestin (red) are seen at 34 days of differentiation (right). Although each system has unique advantages for modeling human brain disorders, hPSC-derived *in vitro* models provide unprecedented platforms for elucidating disease mechanism, biomarker identification, drug screening and toxicity studies, and eventually designing personalized treatments. Scale bar: 100 microns except for the image with iPSC cells immunolabeled with OCT3/4 and SSEA4 (10 microns).

**Table 1: Sample size and functional assays in selected studies using bulk and/or scRNA-seq**

Study	Organoid type	hPSC lines	Bulk /scRNA-seq	Sample size in bulk or scRNA-seq	Disease modeling	Major functional assay
Camp et al., 2015	Self-organized	2 controls	scRNA-seq (Fluidigm system)	333 cells (5 organoids in 5 time points)	Not applicable	Not applicable
Pasca et al., 2015	hCS	7 controls	Bulk	3 lines at 2 time points	Not applicable	2-D calcium imaging, slice & 3-D electrophysiology
Qian et al., 2016	Forebrain; Midbrain and Hypothalamic organoid	4 controls	Bulk	3 samples at 3 time points and 2 samples at 1 time point	ZIKV	Slice calcium imaging & electrophysiology; Proliferation
Xiang et al., 2017	hCO; hMGEO	3 controls	Bulk & scRNA-seq (Chromium system)	Bulk: not clear scRNA-seq: 59,235 cells (2 replicates at two time points)	Not applicable	3-D Calcium imaging; slice electrophysiology
Birey et al., 2017	hCS; hSS	7 controls; 7 patients	scRNA-seq (BD Resolve system & Smart-seq2)	11,838 cells at one time point from multiple organoids	Timothy syndrome	2-D & 3-D calcium imaging; slice electrophysiology
Sloan et al., 2017	hCS	4 controls	Bulk & scRNA-seq (Smart-seq2)	Bulk: 3-15 hCSs per experiment; scRNA-seq: 710 cells (3-7 hCSs per time point; 2 lines and 5 time points)	Not applicable	2-D calcium imaging, cytotoxicity assay
Quadrato et al., 2017	Self-organized	1 control	scRNA-seq (Drop-seq)	scRNA-seq: 80,000 cells (31 organoids; multiple batches)	Not applicable	3-D electrophysiology (high-density silicon microelectrodes)
Watanabe et al., 2017	Human cortical and ventral telencephalic organoid	4 controls	Bulk	12 samples (3 replicates from 4 time points)	ZIKV	3-D Calcium imaging; Slice electrophysiology
Giandomenico et al., 2019	ALI-CO	2 controls	scRNA-seq (10x genomics)	13,280 cells from 6 ALI organoids of 2 hCOs	Not applicable	3-D MEA; Slice electrophysiology
Xiang et al., 2019	hCO; hMGEOs; hThO	2 control	scRNA-seq (Chromium system)	10-12 hThOs (11, 277 cells at 2 time points)	Not applicable	3-D calcium imaging; slice electrophysiology
Velasco et al., 2019	Self-organized;	4 controls	scRNA-seq (10x)	166,242 cells from 21 organoids (at least 3	Not applicable	Not applicable

hCS: human cortical spheroid; hSS: human subpallium spheroid; hCO: human cortical-like organoid; hMGEO: human medial ganglionic eminence (MGE)-like organoid; hThO: human thalamus-like brain organoid; ALI-CO: air-liquid interface cerebral organoids; MEA: multielectrode arrays; 2-D: dissociated cells from organoids; 3-D: intact organoids.



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