Generating 3D Cerebral Organoids From Human Pluripotent Stem Cells to Model Cortical Development and Disease

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Introduction

The mammalian cerebral cortex stands uncontested as the most complex region of the CNS, being composed of billions of neurons and glia whose subtype-specific classification remains to this day incomplete. Using model organisms such as mice, great progress has been made toward understanding how the cortex develops and the logic that shapes its cellular diversity. However, the study of both development and disease of the human cortex is hampered by the limited availability of primary tissue as well as differences in brain development and function between humans and standard animal models. For example, entire regions of the human cerebral cortex are not present in mice, and evidence indicates that therapeutics that show promise in rodents often fail to reproduce the predicted beneficial effects when tested in humans (Institute of Medicine, 2013).

Protocols have recently become available to generate brain "organoids": multicellular, three-dimensional (3D) structures resembling developing human brain tissue that can be derived via self-assembly of cells derived from induced pluripotent stem cells (iPSCs) (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Although much work is still needed to characterize this system, emerging data suggest that cerebral organoids recapitulate aspects of human cortical development, including the generation of some of the latter's cellular diversity and complex tissue architecture. Cerebral organoids therefore represent a potentially important model for studying human cortical development and disease *in vitro*.

In this course, we will cover the advances made in generating 3D brain organoids from pluripotent stem cells, compare and contrast events of organoid formation with embryonic development of the brain, and consider applications for modeling and understanding human brain disease. A particular focus will be placed on the use of brain organoids to model function and dysfunction of the cerebral cortex.

The Mammalian Cerebral Cortex: Elements of Organization and Neuronal Diversity

The neocortex processes information that regulates higher-order functions, including cognition, sensory perception, regulation of fine motor skills, and, in humans, language. These complex behaviors are centrally executed by two major groups of neurons: excitatory projection neurons (PNs) and inhibitory interneurons (INs), both present in a plethora of subtypes (Greig et al., 2013; Kepecs and Fishell, 2014; Lodato et al., 2015). Excitatory PNs are born from neural progenitors located in the developing proliferative zones of the dorsal telencephalon; they are glutamatergic and send long-distance axons to targets within and outside the cortex (Greig et al., 2013; Lodato et al., 2015). The activity of PNs is finely modulated by cortical INs, which are generated from neural progenitors residing in the ventral telencephalon (Anderson et al., 1997) and display a great diversity of molecular signatures, electrophysiological properties, connectivity, and synaptic dynamics; INs are GABAergic and connect locally within the cortical microcircuitry (Kepecs and Fishell, 2014).

The mammalian cerebral cortex is organized radially into six layers (lamina) and horizontally into multiple functional areas (Greig et al., 2013) (Fig. 1). Distinct PN subtypes can be recognized and canonically classified based on the laminar position of their cell bodies, somatic and dendritic morphology, electrophysiological properties, and above all, axonal connectivity (Migliore and Shepherd, 2005; Lodato et al., 2015) (Fig. 2). Indeed, PNs derive their classic nomenclature from their axonal targets and can be broadly classified into intracortical PNs (commissural and associative PNs) and corticofugal PNs (corticothalamic and subcerebral PNs). Intracortical neurons, although present in all six cortical layers, reside in larger numbers in the upper cortical layers (L2/3) and extend axons across the midline to the opposite hemisphere. The majority of intracortical neurons project to contralateral targets via the corpus callosum and are thus dubbed "callosal PNs" (CPNs), whereas a smaller percentage projects via the anterior commissure, the most ancient commissure of the brain. Commissural neurons have been identified in all areas of the neocortex, where they are responsible for integrating bilateral information between homologous areas of the two cerebral hemispheres (Lodato et al., 2015). Neurons projecting contralaterally through the anterior commissure are located mainly in the most lateral cortical areas, which are part of the olfactory–limbic system (Aboitiz and Montiel, 2003). Associative PNs extend axons within the same cortical hemisphere. They can project to either short-distance targets or long-distance targets, in the frontal cortex for example.

We have previously defined the early molecular signatures of CPNs (Arlotta et al., 2005; Molyneaux et al., 2009, 2015; Lodato et al., 2015). While these neurons express a complex, temporally regulated combinations of genes, in this short course, we will

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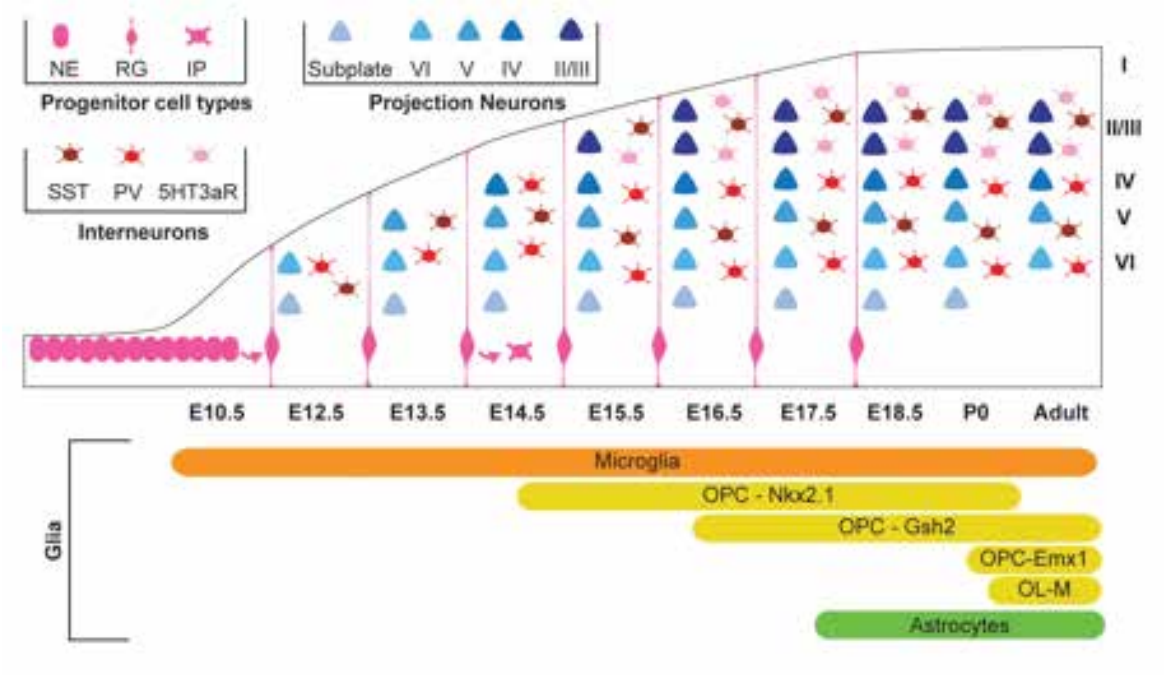

Figure 1. Schematic representing the development of the cerebral cortex of the mouse. Mammalian cortical development occurs along a developmental timeline in which diverse subtypes of both neurons and glia are produced in a defined sequence. OPC, oligodendrocyte precursor cell. Adapted with permission from Harris et al., 2015, their Fig. 2.

often identify them by the expression of *Cux1*, *Cux2*, *Satb2*, *Inhba*, *Lpl*, *Tle3*, and *Hspb3*.

Corticofugal PNs (CFuPNs), located in the deep layers of the cortex (L5 and L6), send axons to distal targets outside the cortex. Corticothalamic PNs are a heterogeneous group of neurons that target different nuclei of the thalamus, while subcerebral PNs (ScPNs) extend axons to multiple targets below the brain, most prominently connecting the cortex to the nuclei of the brainstem and the spinal cord. ScPN somas are found in L5b (across different cortical areas), and different subgroups of ScPNs extend axons to distinct anatomical and functional targets. ScPNs include the corticospinal motor neurons that connect to the spinal cord, the corticopontine PNs that connect to the brainstem motor nuclei, and the corticotectal PNs that project to the superior colliculus (Lodato et al., 2015). We have defined the early molecular signatures of CFuPNs (Arlotta et al., 2005; Lodato et al., 2015; Molyneaux et al., 2015). Like CPNs, these neurons express a complex, temporally regulated combination of genes; in this short course, we will often identify them by the expression of *Ctip2*, *Fezf2*, *Tle4*, *ER81*, *Foxp2*, and *EphB1*.

Cortical INs represent approximately 20–30% of the total number of cortical neurons and make local connections within the cortex (Anderson et al., 2002; Fishell and Kriegstein, 2005; Brandão and Romcy-Pereira, 2015). INs of the cortex are extremely diverse, and their classification is still incomplete. Cortical GABAergic IN subtypes differ in morphology, molecular identity, firing properties, and patterns of local connectivity (Markram et al., 2004).

At a superficial level, three comprehensive and nonoverlapping groups of INs can be found in the neocortex. They express one of three markers: parvalbumin (PV), somatostatin (SST), or the ionotropic serotonin receptor 5HT3a (5HT3aR) (Marín et al., 2012; Kepecs and Fishell, 2014). PV-positive and SST-positive INs are found primarily in the deep layers of the cortex, and 5HT3aR-positive INs preferentially populate the upper layers (Lee et al., 2010).

Within these three classes, many other subtypes can be identified based on the morphology of the soma, axons, and dendrites and their electrophysiological properties. For example, PV-positive INs include fast-

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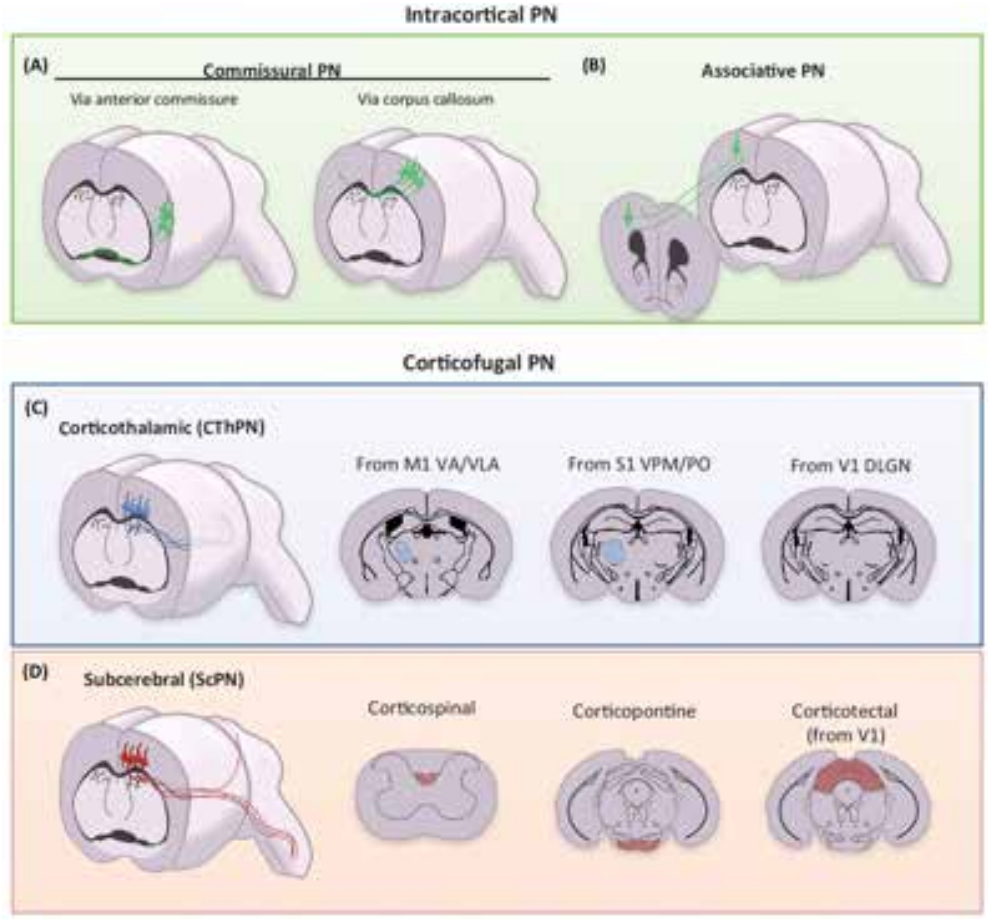

Figure 2. Cortical PN classification by connectivity. PNs are broadly divided into intracortical PNs and corticofugal PNs. Intracortical PNs are further subdivided into (***A***) commissural PNs, which project to the contralateral hemisphere, and (***B***) associative PNs, which project to cortical areas within the same hemisphere. Some commissural PNs connect through the corpus callosum (callosal PNs), while others project via the anterior commissure (***A***). Corticofugal PNs project to subcortical targets and are further divided into (***C***) corticothalamic PNs, subtypes of which project to distinct thalamic targets, and (***D***) subcerebral PNs, subtypes of which send primary axons to the spinal cord, pontine nuclei of brainstem, or midbrain optic tectum. DLGN, dorsal lateral geniculate nucleus; M1, primary motor cortex; PO, posterior nucleus; S1, somatosensory cortex; VA, ventral anterior nuclei; VLA, anterior ventral lateral nuclei; V1, visual cortex; VPM, ventral posterior medial nucleus. Adapted with permission from Lodato et al., 2015, their Fig. 1.

spiking INs belonging to two main morphological classes: large basket cells (which make synapses on the proximal dendrites and the somas of target PNs) and chandelier cells (which target the initial axonal segment of PNs). For a precise classification of cortical INs, we refer the reader to excellent reviews, such as those of Petilla Interneuron Nomenclature Group et al. (2008) and DeFelipe et al. (2013). A variety of markers exist that distinguish distinct classes of INs with varying degrees of specificity and in a temporally restricted manner. In this short course, we will use the following molecular markers to identify cortical INs: *Lhx6*, *5HT3aR*, *PV*, *SST*, *NPY*, *VIP*, and *CR*.

In addition to neurons, the cortex contains a variety of support cells of which there are three major types. Oligodendrocytes are responsible for forming the insulating myelin sheath around the axon of projection neurons, which is required for their proper electrophysiological function (Bercury and Macklin, 2015). Astrocytes, which in the mammalian cortex outnumber neurons, are a highly heterogeneous population of cells that performs an equally broad array of tasks: from assisting in the formation and remodeling of synapses to scavenging and removing extracellular ions and neurotransmitters (Schitine et al., 2015). Microglia are macrophage-like cells responsible for immune surveillance and injury repair

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as well as synaptic pruning and remodeling (Harris et al., 2015; Michell-Robinson et al., 2015).

A Tale of Mice and Men: Progenitors in the Cerebral Cortex of Mice and Humans

The mouse cortex differs significantly from that of humans, so modeling the cellular variety of the human neocortex has been challenging, both *in vivo* and *in vitro*. Thus, the current understanding of human cortical development is limited largely to analyses of postmortem samples of human fetal cortex. For an in-depth treatment of the development and evolution of the human cerebral cortex, we refer the reader to excellent reviews, such as those of Taverna et al. (2014), Geschwind and Rakic (2013), and Florio and Huttner (2014). We will limit ourselves here to a short comparison of cortical progenitor classification and biology between mice and humans because of their relevance to human diseases (e.g., microcephaly) that have been modeled using human cerebral organoids.

The human cortex is vastly expanded in surface area relative to its size, producing a gyrencephalic rather than a smooth, lissencephalic cortex, as in mice (Borrell and Gotz, 2014). It also contains a much increased diversity of cellular types and distinct functional areas. The increase in size and cellular diversity of the human cortex is supported at least in part by an expanded subventricular zone (SVZ), which contains a vastly increased number and diversity of progenitors (De Juan Romero and Borrell, 2015). In mice, after neural tube closure, neuroepithelial (NE) cells with stem cell–like properties initially divide symmetrically to expand the progenitor pool. Later, they differentiate into more restricted progenitors known as radial glial cells (RGCs), which are bipolar cells with radial fibers contacting the apical ventricular zone and the pial surface. RGCs serve as a scaffold for neuronal migration, and they are also multipotent progenitor cells able to generate neurons, astrocytes, and oligodendrocytes (Malatesta et al., 2000; Anthony et al., 2004). At the onset of neurogenesis, the majority of RGCs exhibit asymmetric divisions in the ventricular zone to produce an RGC daughter cell and either a neuron or an intermediate precursor cell (IPC) (Pontious et al., 2008). IPCs then migrate basally to form the SVZ, where they further divide symmetrically to give rise to two to four neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). The progenitor composition of the human developing cortex is more complex. One key distinction of the SVZ of humans (and that of primates, more broadly) is that, in addition to increased numbers of IPCs, it contains an expanded new population of progenitor cells named outer radial glia (oRG), which lack apical contacts but retain a basal process to pia (Hansen et al., 2010). Interestingly, oRGs are also present in mice but at a very low frequency (Wang et al., 2011). A striking difference between oRGs in humans and mice is that murine oRGs directly produce neurons by symmetric division, whereas oRGs in humans divide asymmetrically to self-renew and generate a self-amplifying IPC, which then generates neurons (Hansen et al., 2010; Wang et al., 2011; LaMonica et al., 2013). Thus, oRG cells might contribute to the increased number and tangential dispersion of human neurons and to cortical folding (Sun and Hevner, 2014). Recent studies in primates have also shown that, in addition to IPCs, at least four types of oRG cells are present in the SVZ, contributing to increased progenitor diversity (Betizeau et al., 2013).

Modeling Human Cortical Development With Stem Cell–Derived Cerebral Organoids

Research using embryonic stem cells (ESCs) and iPSCs has demonstrated a surprising capacity of initially homogenous cultures to spontaneously self-assemble under permissive conditions into complex structures resembling endogenous tissue (Sasai, 2013). The Sasai laboratory first described an ESC aggregation protocol for producing relatively simple assemblies of cortical-like neurons (Eiraku et al., 2008); subsequently, using a modified culture protocol, they were able to generate tissue resembling cortical neuroepithelium (Nasu et al., 2012). Most strikingly, spontaneously self-assembling 3D optic-cup structures could be generated by these methods from both mouse (Eiraku et al., 2011) and human (Nakano et al., 2012) ESCs. These ESCs undergo a morphogenetic process resembling endogenous optic-cup development and subsequently form laminated retina tissue containing major classes of neural retinal cell types. Similar ESC-derived or iPSC-derived "organoids" have been produced for a variety of other tissues, including intestine and pituitary adenohypophysis (Sasai, 2013).

In vitro differentiation of stem cells to cortical neurons was first reported several years ago (Watanabe et al., 2005; Eiraku et al., 2008; Gaspard et al., 2008). Recently, methods have been adapted to create more complex, 3D structures in which iPSCs, triggered to differentiate into neuroectoderm

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by short-term application of appropriate growth factors, spontaneously differentiate further and self-assemble into a 3D organoid (Fig. 3). This organoid mimics some properties of the developing embryonic brain, including the presence of radial glia–like stem cells and neurons that appear to recapitulate the migration and intercalation that occur during embryonic cortical development (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Much work remains to be done to understand the extent to which 3D cerebral organoids mimic the architecture, developmental trajectory, and cellular diversity of the developing human cortex; however, initial data indicate a significantly improved system for recapitulating these key steps of development in the dish.

This system has several unmet goals and limitations that will be discussed in the course. For example, it is clear that organoids currently reflect the cellular composition of the embryonic human brain, and thus, a major unmet goal is to produce tissue that resembles the postnatal human brain. This is not a trivial task, as native human cortical development covers several years of prenatal and postnatal development. Although organoids are amenable to long-term culture (Lancaster et al., 2013, reported maintaining healthy cultures for more than 10 months), they lack blood vessels to support tissue oxygenation and appear to exhaust their capacity to grow and develop past early embryonic stages. In addition, it is unclear whether the organoids contain the full range of neuronal and glial populations found in the embryonic brain, although studies are ongoing to address these limitations. In addition, the question of whether circuit organization within organoids resembles that of endogenous tissue has not been investigated.

Nonetheless, the organoid system holds great promise for studying basic developmental biology of the human cortex because it allows ready access to

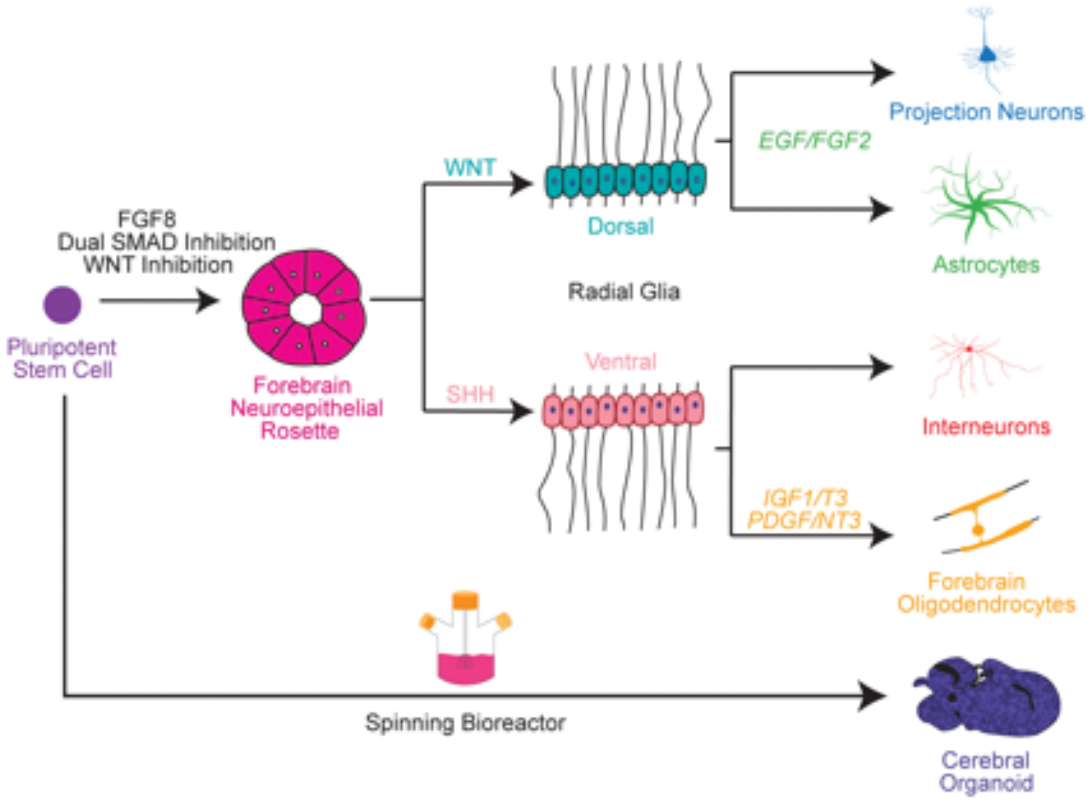

Figure 3. Schematic representing the generation of cortical cell types from pluripotent stem cells *in vitro*. Traditional methods (top) use sequential application of growth factors to promote differentiation of predetermined cell types. The organoid method (bottom) exploits the self-organizing ability of induced neuroectodermal cells to form an organized, multilayer structure containing multiple differentiated cell types. EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; NT3, neurotrophic factor 3; PDGF, platelet-derived growth factor; SHH, sonic hedgehog. Adapted with permission from Harris et al., 2015, their Fig. 3.

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cells for comparative study with human fetal tissue. In particular, transcriptome and proteome analyses will benefit from an on-demand supply of fresh, healthy cells. In addition, both the starting iPSCs and the organoids themselves can be genetically manipulated to introduce, for example, cell type–specific fluorescent markers to trace cell populations, or disease-causing mutations to dissect molecular mechanisms of development and disease.

Lancaster et al. (2013) have already demonstrated, in a proof-of-concept experiment, the use of patient-derived iPSCs to model microencephaly, a condition characterized by an abnormally small brain. Although causative mutations of this condition have been identified, mouse models do not recapitulate the patient phenotype, possibly because of the difference in human versus mouse progenitor behavior. Using the organoid system, Lancaster et al. observed a reduction in early neural stem cell populations along with premature neural differentiation, suggesting that the *CDK5RAP2* mutation found in patients disrupts the timing of the switch between proliferative and neurogenic divisions in neural progenitors. Similar experiments can be imagined for a variety of human diseases, including complex polygenic diseases and those for which the causative mutations have not been identified.

More recently, a modified protocol for organoid generation has been used by Mariani et al. (2015), who studied neurodevelopmental defects in organoids derived from patients with severe idiopathic autism spectrum disorder (ASD). These ASD-derived organoids exhibited enhanced proliferation of progenitors and overproduction of GABAergic inhibitory neurons, leading to phenotypes consistent with studies of patient-derived tissue. Mariani et al. further demonstrated that abnormally high expression of the transcription factor FOXG1 plays a part in these differences (Mariani et al., 2015).

These studies provide promising initial examples of the use of organoids not only to study normal human brain development but also to model human diseases that have a developmental origin. In this course, we will review different methods for generating human cerebral organoids from pluripotent stem cells. We will compare and contrast available protocols and consider the limitations and advantages of this system for studying cortical development and disease.

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