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Cell diversity in the human cerebral cortex: from the embryo to brain organoids

Paola Arlotta[1,2] and Sergiu P Paşca[3,4]

The development and wiring of the central nervous system is a remarkable biological process that starts with the generation of and interaction between a large diversity of cell types. Our understanding of the developmental logic that drives cellular diversification in the mammalian brain comes, to a large extent, from studies in rodents. However, identifying the unique cellular processes underlying primate corticogenesis has been slow, due to the challenges associated with directly observing and manipulating brain tissue from these species. Recent technological advances in two areas hold promise to accelerate discovery of the mechanisms that govern human brain development, evolution, and pathophysiology of disease. Molecular profiling of large numbers of single cells can now capture cell identity and cell states within a complex tissue. Furthermore, modeling aspects of human organogenesis *in vitro*, even for tissues as complex as the brain, has been advanced by the use of three-dimensional organoid systems. Here, we describe how these approaches have been applied to date and how they promise to uncover the principles of cell diversification in the developing human brain.

Addresses
[1] Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA
[2] The Stanley Center for Psychiatric Disease at the Broad Institute of MIT and Harvard, Cambridge, MA 02138, USA
[3] Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA 94305, USA
[4] Stanford Human Brain Organogenesis Program, Stanford Wu Tsai Neurosciences Institute, Stanford, CA 94305, USA

Corresponding authors: Arlotta, Paola (paola_arlotta@harvard.edu), Paşca, Sergiu P (spasca@stanford.edu)

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Introduction

During development, a process known as organogenesis progressively builds the tissues and organs of the body. Organogenesis is under heavy evolutionary constraint, such that every time development takes its course, each embryo acquires the same compendium of cell types, positioned in a similar tissue architecture, to ultimately produce anatomically defined and functionally integrated organs. This fascinating series of events has been studied for centuries in an attempt to acquire a mechanistic understanding of both the healthy and diseased organism. These efforts, combined with recent progress in directing the fate of pluripotent stem cells *in vitro* [1–3], has led to the somewhat provocative question of what aspects of the elaborate process of human organogenesis can be recapitulated outside the embryo [4]. This issue becomes particularly significant when one considers that certain human tissues simply cannot be accessed experimentally, and thus their development, functionality, and disease remain understudied.

Perhaps no other organ embodies these limitations as uniquely as the brain [1,5]. More specifically, building experimental models of the human brain has been challenging for at least three reasons. First, while studies of brain development in other species, such as rodents, continue to inform us on developmental mechanisms shared among mammals, the human brain is different even from that of our closest primate relatives [6,7]. Conversely, there is very limited access to developing human tissue due to obvious ethical considerations [8]. Given the sheer length of human brain development, rare tissue biopsies can only provide a snapshot. Perhaps most notably, recent investigation of neuropsychiatric disorders highlights a complex genetic architecture that may include the combined contribution of large numbers of variants of small effect or rare genetic events of variable penetrance [9]. Studying the biology of these complex disorders requires the context of the human genome, and thus human cellular models become evident as an essential experimental platform.

Advances in stem cell biology, combined with existing basic knowledge of brain development, has paved the way for a very promising area of neuroscience: self-organizing, three-dimensional cultures of *in vitro*-produced brain tissue, commonly referred to as brain organoids. These preparations can be thought of as a reductionist system with the potential to mimic aspects of the cellular composition, development, and activity of the brain. Here, we review recent work on human brain organoids with a particular focus on how these systems may help investigation and understanding of the principles that lead to cellular diversification in the human cerebral cortex.

Organoids as experimental models of the developing human brain

Brain organoids are self-organizing 3D cultures that can be generated from human pluripotent stem cells, either by undirected differentiation methods that omit inductive signals, or by patterning through directed differentiation methods that guide the formation of region-specific brain organoids or spheroids (reviewed in Refs. [4,5,10–12]). For example, this approach can be used to generate specifically the dorsal forebrain [13,14], which later generates the cerebral cortex; forebrain organoids with dorsal or ventral identities [15••,16,17]; midbrain organoids [18]; or cerebellar organoids [19]. Once formed, region-specific organoids can be fused together or combined with external cell preparations of various lineages to produce brain assembloids, which enables engineering together more than one brain region or incorporating cells that would not spontaneously arise within a given organoid system [20].

Pioneering work over the last decade has demonstrated that a powerful process of self-organization of pluripotent stem cells grown in three-dimensional cultures enables remarkable differentiation and cytoarchitectural arrangement *in vitro* [21]. Equally importantly, the culture conditions used in three-dimensional models enable long-term maintenance of tissue *in vitro*, beyond what is possible in conventional two-dimensional cultures (reviewed in Ref. [4]). This is important given the protracted duration of human brain development, which extends into postnatal life, and is particularly relevant for the cerebral cortex.

The rich diversity of cell types that emerge during mammalian corticogenesis *in vivo* are largely produced from two main germinal zones, located in the ventral and dorsal telencephalon [7,22,23]. Pyramidal excitatory neurons are born from progenitors located in the dorsal telencephalon, and give rise, in a stereotypical temporal sequence, to many individual classes of neurons that generate a structure called the cortical plate. Over time, the cortical plate becomes the layered cerebral cortex, where each layer contains its own unique compendium of neuronal subtypes. A great variety of pyramidal neuron subtypes can be distinguished, which are canonically classified based on the laminar position of their cell bodies, molecular properties, somatic and dendritic morphology, electrophysiological properties, and axonal connectivity. Ultimately, pyramidal neurons will connect the cortex to all of its intra-cortical and subcortical targets across many regions of the CNS (reviewed in Refs. [22,24]). The activity of pyramidal neurons is modulated locally by a large class of inhibitory interneurons, which are born in the ventral forebrain and migrate over great distances to connect with pyramidal neurons and form the local cortical microcircuit [25–27]. Finally, after neurogenesis is largely complete, an extended wave of gliogenesis takes place that generates astrocytes and oligodendrocytes [28]. Much is known about the properties of these diverse cells in the rodent cerebral cortex. However, our knowledge of how the human cerebral cortex is formed is much less complete (reviewed in Refs. [7,23]), given that analysis of the human embryonic brain is largely limited by tissue availability to a narrow, early period of cortical development. Many questions, therefore, remain unanswered about the developmental origin of the cellular constituents of the human cerebral cortex, the molecular logic that drives their developmental trajectories, and, in particular, how cell types differ between the primate and rodent brain. Human brain organoids promise to provide an alternative system to address some of these core questions.

Human brain organoids and assembloids: generating cell diversity outside the embryo

Several studies have investigated the cellular composition of human brain organoids generated through a variety of protocols. In an initial effort to deconstruct cellular heterogeneity in 3D whole-brain organoid cultures, Camp *et al.* used single-cell RNA sequencing of brain organoids differentiated using an undirected approach [29•]. The authors analyzed 508 cells isolated either from whole organoids, or from presumptive cortical regions microdissected from organoids, after two months of differentiation in culture. Bioinformatic clustering of cells indicated the presence of both dorsal and ventral forebrain fates, as well as cycling and non-cycling mesenchymal cells. Importantly, direct comparison to primary tissue revealed significant similarities to human fetal cerebral cortex. In a follow-up study, Mora-Bermudez *et al.* investigated cell composition and lineage relationships in brain organoids derived from human versus chimpanzee pluripotent stem cells [30]. Approximately half of the cells analyzed in these organoids, which were differentiated for up to 80 days, displayed a molecular signature suggestive of cortical origin. Interestingly, this study identified differentially expressed genes in radial glial cells in human versus chimp organoids, which included genes related to intercellular signaling and cell cycle length.

Profiling of thousands of single cells in region-specific organoids that resemble the dorsal forebrain [14] revealed the presence of regionally appropriate cell types, including dorsal radial glia, intermediate progenitors, glutamatergic neurons and astrocytes [31••]. In contrast, profiling of organoids resembling the ventral forebrain demonstrated subpallial progenitors, GABAergic interneurons and astroglial cells [31••]. Subsequent comparisons of cortical organoids across multiple human pluripotent lines and experiments demonstrated high differentiation reliability in cell composition and overall transcriptional signature [32]. Furthermore, closer inspection of the transcriptome of neurons using deep sequencing of single cells showed the presence of cells expressing markers of the deep cortical layers, as well as neurons expressing upper layer markers [33]. As expected,

cells expressing upper layer markers primarily emerged at later stages of *in vitro* differentiation, and their generation was followed by a wave of astrogenesis. Interestingly, over 20 months *in vitro*, glial lineage cells in cortical organoids transition from a predominantly fetal to an increasingly mature astrocyte state in a developmental progression resembling that *in utero*. Notably, region-specific organoids can also be fused together to create tissue containing cell types of different anatomical origins. For instance, assembloids that combine organoids resembling ventral and dorsal regions of the forebrain have been shown to be a valid system to model complex cell behaviors; GABAergic cells migrate from the ventral to the dorsal components of the fused assembloids using movement dynamics that resemble the migration of endogenous interneurons [31••,34,35].

Brain organoids developed using a whole-brain organoid protocol and cultured for up to nine months *in vitro* have been profiled using droplet-based single-cell RNA sequencing of ~80 000 single cells across multiple organoids [36••]. In this study, single organoids were profiled individually and at two developmental ages, to also gain initial knowledge of the molecular developmental trajectories of cell types and cellular composition. This approach demonstrated a broad diversity of cell types emerging in this model, including several late-developing cortical cell types as well as cells of other brain regions and of the retina. Interestingly, these organoids also produced identifiable subclasses of cells within these broad groups (for example, distinct subtypes of excitatory pyramidal neurons), as well as astroglia. Cellular diversification could similarly be observed for retinal cell types, where major subdivisions of retinal neurons and glia were generated. Furthermore, molecular profiling of these cells over a span of six months *in vitro* indicated that cell types were generated in a defined temporal order that reflected endogenous development.

All together, these studies illustrate that fundamental processes of cellular specification and diversification occur in brain organoids, and that organoids have the potential to produce a large variety of cell types and broadly follow appropriate sequential stages of development. This suggests a model whereby genetically encoded instructions are sufficient, at least for cerebral cortex, to drive progenitor cells to generate a compendia of cell types without the context of the embryo. These models also open avenues for disease research; leveraging cell diversity in forebrain organoids has allowed the identification of a mitotic defect in outer radial glia derived from patients with a form of lissencephaly called Miller–Dieker syndrome [37•]. While several obstacles must be overcome, including the need for models in which each individual organoid consistently follows identical processes of cell fate acquisition and diversification, these studies suggest that organoids will likely be productive model systems for elucidating the molecular principles of fate specification and diversification in both the normal and disordered human brain.

Fidelity of differentiation in organoids and parallels with *in vivo* brain development

Progress in understanding the cellular composition and developmental potential of organoids has prompted several fundamental questions. First and foremost, how similar are the cell types produced in brain organoids to those of the fetal or adult human brain? Work on this problem is in its infancy, but the increasing number and volume of single-cell datasets from organoids has enabled direct comparison to single-cell profiling of the human fetal cortex. The message emerging from studies that have compared, in the same experimental setting, cells derived *in vitro* and primary cells isolated from human brain tissue show that cells made in organoids do indeed molecularly resemble their endogenous counterparts [29•,30,33,37•,38,39]. That is not to say that they are identical, but that they can at least partially replicate the distinctive components of the cell type's molecular profile. It is possible that once the initial patterning of progenitors toward a broad brain region, such as the cerebral cortex, has been initiated, fate specification of its cellular constituents largely follows constrained, intrinsic driving principles. It remains to be fully explored whether cell types generated using different organoid protocols share a similar degree of fidelity to the endogenous tissue. Should this be the case, this would further support the idea that fate specification and diversification largely follow intrinsic programs independent of the embryonic context.

The capacity of organoids to generate cell types that molecularly resemble their endogenous counterparts raises the further question of whether the progressive developmental steps that produce these cell classes and subtypes are also reminiscent of those of the embryo, and whether they occur consistently in different organoid models. In principle, any given cell type could be generated using a different molecular and developmental logic than that of the embryo. From a practical viewpoint, resolving this point will be important to establish whether brain organoids can reliably serve as experimental tools to decode the logic that builds the cellular constituents of complex brain regions, such as the human cerebral cortex.

Thus far, most studies of cell-type specific properties in organoids have relied on transcriptional analysis. However, recent work is beginning to shed light on other features present in cells produced in these 3D cellular systems. Once again it appears that the cells acquire functional properties that resemble, in broad strokes, distinctive features of their *in vivo* counterparts. One early example has come from analysis of progenitor cell behavior in organoids. Outer radial glia cells are a type of progenitor cell that is characteristic of the primate brain, and nearly absent from the brain of rodents [40]. These cells have a variety of unique properties relating to their proposed role in the evolutionary expansion of the cerebral cortex in primates. Work on human fetal tissue has uncovered a unique mode of saltatory migration that allows these cells to move quickly over long distances

within the developing human cortex [7,41]. Recent work has shown that outer radial glia are not only generated in organoids [15••,33,37•], but display their distinctive migration kinetics *in vitro* [37•]. This important finding establishes organoids as promising models to investigate this elusive cell type, which, while critically important for the development of the primate cortex, cannot be studied in rodents. Other examples of cells in organoids that acquire appropriate functional traits include retinal cells able to respond to light stimuli [36••], neurons that mature electrophysiologically over time [14,15••] or following migration [31••], myelinating oligodendrocytes [38,39], and organoid-derived astrocytes that progressively develop basic metabolic and phagocytic properties that resemble those of the endogenous cells [33].

More work needs to be done to advance functional maturation and assemble neural circuits within organoids or assembloids, but these findings suggest that fate specification in organoids leads to cells that acquire distinctive type-specific properties, and suggest that brain organoids might be further used to investigate certain functional phenotypes in the context of disease.

Conclusions

It is still the early stages of establishing brain organoid technologies as broadly used and reliable models. However, progress is rapid, and increasingly accurate and reproducible models are being developed. Some of the limitations of these systems are beginning to be addressed; others will require longer-term investigation, but the potential of these models is becoming quite clear. We foresee a series of improvements that will need to be made, including the induction of reliable axis polarization and predictable anatomical organization; the development of more mature (including aging) neuronal and glial cells; and the formation of consistent patterns of connectivity. As single-cell profiling methods and analyses are also advancing rapidly, these combined techniques could be further leveraged to, for example, identify disease phenotypes in patient-derived cultures. The development of higher-throughput methods for *in situ* transcriptomics, which operates on sections of intact tissue, would be particularly relevant for analyses of the structural organization of organoids [42]. Lastly, the ability to combine single-cell RNA sequencing with genomic and chromatin profiling [43], or with genetic or environmental perturbations (e.g. Perturb-seq [44]), will enable organoids to be applied to ever more detailed studies of both development and disease. Moving forward, brain organoids and assembloids, in combination with single cell profiling and cutting-edge imaging and functional interrogation methods, have the potential to revolutionize the way we understand human brain development and evolution, and to accelerate the pace of discovery in human neuropsychiatric disorders.

Conflict of interest statement

Stanford University has filed several patent applications that cover the generation and assembly of region-specific neural spheroids/organoids.

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