Chapter 8
Brain Organoids: Expanding Our Understanding of Human Development and Disease

L. B. Chuye, A. Dimitri, A. Desai, C. Handelmann, Y. Bae, P. Johari, J. M. Jornet, I. Klejbor, M. K. Stachowiak, and E. K. Stachowiak

Abstract Stem cell-derived brain organoids replicate important stages of the prenatal human brain development and combined with the induced pluripotent stem cell (iPSC) technology offer an unprecedented model for investigating human neurological diseases including autism and microcephaly. We describe the history and birth

L. B. Chuye, A. Dimitri, A. Desai, and C. Handelmann are co-first authors.

L. B. Chuye · A. Desai · C. Handelmann · Y. Bae
Department of Pathology and Anatomical Sciences, State University of New York, Buffalo, NY, USA

A. Dimitri
Department of Pathology and Anatomical Sciences, State University of New York, Buffalo, NY, USA

Department of Biological Sciences,, State University of New York, Freedonia, NY, USA

P. Johari · J. M. Jornet
Department of Electrical Engineering, State University of New York, Buffalo, NY, USA

I. Klejbor
Medical University of Gdansk, Gdańsk, Poland

M. K. Stachowiak
Department of Pathology and Anatomical Sciences, State University of New York, Buffalo, NY, USA

Western New York Stem Cells Culture and Analysis Center, State University of New York, Buffalo, NY, USA

Department of Biomedical Engineering, State University of New York, Buffalo, NY, USA

E. K. Stachowiak (✉)
Department of Pathology and Anatomical Sciences, State University of New York, Buffalo, NY, USA

Western New York Stem Cells Culture and Analysis Center, State University of New York, Buffalo, NY, USA
e-mail: eks1@buffalo.edu

L. Buzanska (ed.), *Human Neural Stem Cells*, Results and Problems in Cell Differentiation 66, https://doi.org/10.1007/978-3-319-93485-3_8

of organoids and their application, focusing on cerebral organoids derived from embryonic stem cells and iPSCs. We discuss new insights into organoid-based model of schizophrenia and shed light on challenges and future applications of organoid-based disease model system. This review also suggests hitherto unrevealed potential applications of organoids in combining with new technologies such as nanophotonics/optogenomics for controlling brain development and atomic force microscopy for studying mechanical forces that shape the developing brain.

Abbreviations

AFM	Atomic force microscopy
CNVs	Copy number variations
ESCs	Embryonic stem cells
INFS	Integrative nuclear FGFR1 signaling
iPSCs	Induced pluripotent stem cells
NCCs	Neural committed cells
nFGFR1	Nuclear fibroblast growth factor receptor-1
NPCs	Neural progenitor cells
SNPs	Single nucleotide polymorphisms

8.1 Expanding Our Understanding of Human Neurological Disease

From the early descriptions of epilepsy found in the book of Charaka Samhita (1500– 500 BCE) to Broca's aphasias discovered by Pierre Paul Broca during the 1800s (Lazar and Mohr 2011), to the more recently described phantom limb syndrome, various neurological diseases have always captivated human imagination. Yet, for all the predicament they cause to human kind, and despite all the remarkable progress in technology, advances in this area are hard to come by. Truthfully, the human brain with its anatomical complexity and connectivity is a more abstruse system to study; arguably though, part of the problem in understanding its intricate ways dwells in the fact that scientists lack appropriate models to study it (Quadrato et al. 2016).

Studies in common rodent models, phylogenetic differences aside, have provided us a head start and have prompted us to elucidate the basic layout of the mammalian brain. As some researchers have indicated, critical differences between species ought to be taken into consideration when modeling a human disease into an animal system (Muotri 2016; Seok et al. 2013; DeFelipe et al. 2002). For instance, Quiñones-Hinojosa and Chaichana (2007) have demonstrated substantial differences between the cortical organization of human and rodent brains, particularly in the subventricular zone (SVZ) and layer II of the cortex. Located laterally in the wall of the ventricles, the SVZ is one of the main neurogenic areas in the mammalian

brain. The SVZ of the rodents though has a particular rostral migratory stream that runs parallel to the basal ventricular surface, for which there is no known analog in humans. In the same vein, layer II in humans is a hypocellular area, but this layer in rodents is tightly packed with astrocytes. DeFelipe et al. (2002) have reported other significant differences between clades. They discovered double bouquet interneurons present in humans and macaques, which has not been detected in rodents (DeFelipe et al. 2002). This study also demonstrated differences in the neuronal migratory patterns between species (DeFelipe et al. 2002). As Marin et al. (2001) described, up to 65% of the GABAergic (Dlx1/2+ and Mash1+) neurons in primates originate from the ventricular and subventricular dorsal telencephalon ,while the other 35% (Dlx1/2+ and Mash1−) of neurons originate in the ganglionic eminence of the ventral telencephalon (Marin et al. 2001). Yet, most of the GABAergic neurons in rodents originate in the ganglionic eminence of the ventral telencephalon (Anderson et al. 1999; Tan et al. 1998). In addition, the GABAergic neurons make up 15% of all cortical neurons in rodents, but they are more abundant in the primate cortex, where they constitute approximately 25% of all neurons (DeFelipe et al. 2002). This observation suggested a more prevalent inhibitory signaling in the primate cortex. Researchers have also found that the percentage of asymmetrical synapses, on which the postsynaptic density is prominent, was higher in human cortex compared to rodents, particularly in layers IV, V, and VI (Peters and Palay 1996; Peters et al. 1991).

The effects of pathologically altered proteins, which in humans cause diseases, may vary between species. In an exemplary study, Thomas et al. managed to recreate mutations of DNA repair exonuclease 1 (TREX1) in a mouse model, which in humans leads to the Aicardi–Goutiéres Syndrome (Thomas et al. 2017). In humans, the TREX1 mutations cause microcephaly, intracerebral calcifications, and global developmental delay (Abdel-Salam et al. 2017). Surprisingly, mice with the same mutations failed to develop significant neurological manifestations.

8.2 From Cancer and Epilepsy to Neural Stem Cells

Cognizant of the limitations posed by phylogenetic differences, many researchers have argued in favor of transitioning toward using more apparent human systems (Quadrato et al. 2016; Muotri 2016). In the last couple of decades, emergence of new technologies has enabled scientists to isolate and manipulate human stem cells to grow 3D organ-like structures, including cerebral-like organs. However, the connection between the stem cells and the brain had begun long before these technologies were even conceived.

Altman et al. and Nottebohm et al. reported the occurrence of adult neurogenesis in rats, cats, and birds' brains as early as 1962 and 1985 (Altman and Das 1965; Nottebohm 1985). Subsequently, other groups had tried to identify, to no avail, stem cells in the adult brains of higher primates (Rakic 1985; Eckenhoff and Rakic 1988). The publication of two key papers in the field of neuroscience would eventually

change and advance this field. By 1994, Kirschenbaum and Goldman were able to culture human brain stem cells in vitro (Kirschenbaum et al. 1994). The brain samples for their research were obtained from 11 patients from the age of 15 to 52 who underwent anterior temporal lobectomy. Out of the 11 sets of brain specimens, several contained ventricular tissues. By culturing these disassociated tissues, the researchers were able to identify fiber-projecting neuron-like cells. Some of these cells were pulse-prelabeled with 3′H-thymidine and displayed depolarization-induced calcium fluxes typical to mature neurons. These observations indicated that neurogenesis was taking place. These significant findings were not broadly acknowledged by the scientific community until 1998, when Eriksson et al. discovered in vivo neurogenesis occurring in the adult human brain (Eriksson et al. 1998). In this study, Eriksson used a nonradioactive bromodeoxyuridine (BrdU), a synthetic analog of thymidine, to monitor neuronal proliferation in terminal cancer patients. Five patients between the ages of 52 and 72 accepted to participate in this study. The brain autopsy samples were collected from the hippocampal area, and Eriksson ensured no sign of metastasis in any of the brains sampled. The tissue samples were fixed, sectioned, and immunostained with anti-BrdU antibody. The results showed that all the samples obtained from patients treated with BrdU were undergoing mitosis. This is how, thanks to those generous epileptic and cancer patients who donated their brains to science, scientists were able to demonstrate that neurogenesis in the brain of adult human indeed occurred. This is also how the long-standing Santiago Ramon y Cajal's dogma of "Everything may die, nothing may be regenerated" came to an end (Stahnisch and Nitsch 2002).

8.3 From ESCs to iPSCs

The discovery of the neural stem cell in adult human brain led to a renaissance in the fields of neuroscience and disease. There was no controversy about the use of the brain stem cells for research. As long as the patients and their families were informed about the risks and had their consent, scientists were able to obtain and use those cells in their laboratories (Eriksson et al. 1998; Kirschenbaum et al. 1994). However, the problem was with the scarcity of the brain-derived neural stem cells, and, as latter learned, they were not a suitable starting material for developing cerebral organoids.

This problem was overcome by using the embryonic stem cells (ESCs) and learning how to differentiate these pluripotent cells into the multipotent neural stem cells. The human ESC (hESC) lines provided ample material for developing organoid technologies, but they were not universally admitted across the world scientific community. Presenting the arguments in favor or against the use of hESCs for research makes for a lengthy discussion and far outreaches the objectives of this review. However, a breakthrough occurred in 2006 when Takahashi and Yamanaka were able to reprogram and induce pluripotency in differentiated cell lineages (Takahashi and Yamanaka 2006). By infecting human fibroblasts with retroviruses expressing few transcription factors such as Oct3/4, Sox2, Klf4, and

c-Myc, Yamanaka group was able to prime the fibroblasts to dedifferentiate and express markers of the pluripotent stem cells. The reprogrammed cells were referred to as the induced pluripotent stem cells (iPSCs). A similar mechanism was used by herpesviruses to hijack the host molecular machinery in order to synthetize their own viral proteins and to proliferate (McBride 2017; Sadeghipour and Mathias 2017). Takahashi and his colleagues were also able to reprogram the iPSCs into becoming neural stem cells and cardiomyocytes using protocols developed earlier by Kawasaki et al. (2000) and Laflamme et al. (2007).

Natural follow-up questions emerged after Takahashi's discovery: Are there any differences between the ESCs and the iPSCs? Are there any advantages in working with one versus the other? Like ESCs, iPSCs can express stem cell markers and can derive three germ layers, but despite the similarities, small difference does exist. By comparing the reported transcriptional profiles of different human iPSCs and ESCs available on the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/), Ghosh et al. found few differences, which in part could reflect different analytical methodologies (Lee et al. 2013). The results suggested that younger the tissue of origin, potentially shorter the distance between the iPSCs and ESCs.

8.4 The Birth of the Organoids

In this section, we will briefly review some of the techniques used by different groups to grow human tissues, focusing in particular on the recent developments of cerebral organoids. At the end of the section, we will present brief annotations on the importance of adopting these new technologies as a surrogate model for the study of human embryology, human diseases, for drug development, and the implications this new technology might have for the future of medicine.

Tissue culture techniques were devised more than a century ago to study animal cells. The early approach was to observe aggregated tissue samples. The methods of disaggregation and replating of tissue cells were developed later on (Carrel 1912; Carrel and Burrows 1911; Freshney and ebrary Inc. 2010). In 1952 as Renato Dulbecco was trying to grow viruses in a petri dish on a bed of fibroblasts, he found that adding trypsin would detach cells from the dish. Then, he reseeded these free fibroblasts in the new dish, which would repopulate by forming a monolayer of single cells (Dulbecco 1952). If the cells are allowed to reaggregate, in agar, in a collagen matrix, or in other media, the resulting 3D formation is referred to as a histotypic culture, in which the aggregating cells belong to the same type and lineage. In the organotypic culture, on the other hand, the aggregating cells belong to different types/lineages that form an organ. Organotypic cultures are useful for studying the relations among different cells (Abdel-Salam et al. 2017; Muotri et al. 2005; Narla et al. 2017; Muotri 2016; Ruzzo and Geschwind 2016; Renner et al. 2017; Sutcliffe and Lancaster 2017; Giandomenico and Lancaster 2017; Eiraku and Sasai 2012; Sasai 2013) and prepare the ground for developing organ-like structures,

the organoids. For instance, Dr. Hans Clevers has used organotypic cultures for growing intestinal, liver, stomach, lungs, and prostate organoids (Clevers 2013; Bartfeld and Clevers 2017; Clevers 2009; Barker et al. 2008).

Parallel to the discovery of neurogenesis in the adult human brain, a breakthrough new method for tissue culture occurred during the 1990s. In 1990, Dr. Mina Bissell at the Lawrence Berkeley National Laboratory in California was attempting to grow murine mammary gland tissue in a petri dish. She had transplanted mammary acinus from an early pregnant mouse to the culture dish, but these tissues disorganized and stopped producing milk. Dr. Bissell then modified the microenvironment of the tissue culture by adding extracellular matrix (ECM) extracts and allowing cells to reorganize themselves into a three-dimensional (3D) structure analogous to the acinus. This modification resulted in the production of milk and led to the hypothesis that the microenvironment was activating genomic programs for cell interactions and aggregations to become functional tissues and organs (Foley 2017). This was a seminal finding, ultimately leading to the development and growing of the human organoids.

Early organotypic cultures used to study neural development were referred to as neurospheres (Lancaster and Knoblich 2014; Eiraku and Sasai 2012). The protocol for culturing neurospheres consists of laying NSCs in a nonadhesive substrate and adding mitogenic growth factors such as epidermal growth factor (EGF) and extracellular FGF-2 (Fig. 8.1). Later on, after removing growth factors, the multipotent NSCs begin to differentiate into neuronal, astrocytic, and oligodendrocytic lineages. Although neurospheres self-assemble into structures that resemble simple brain structures, most of the cell lines remain undifferentiated. The neurospheres tend to have clonal specificity toward a particular cell lineage (Gil-Perotin et al. 2013). As Lancaster noted, neurosphere cytostructure lacked the organization when compared with more advanced organoids (Lancaster and Knoblich 2014). Moreover, other researchers have also been growing organotypic culture from tumor cells, to which they refer as tumorspheres (Kaushik et al. 2017; Yang et al. 2017).

An important step in growing cerebral organoids was 2D structures referred to as neural rosettes. They develop from progenitor cells through an anchorage-dependent growth, but its architecture represents early stages of the neural tube organization (Broccoli et al. 2014). The neural progenitors in the rosettes present apicobasal morphology and undergo interkinetic nuclear migration. However, significant limitation to this 2D model was the lack of cell type differentiation that as some researchers have suggested might be related to anchorage dependence characteristic of the neural progenitors (Lancaster and Knoblich 2014). Another type of organotypic culture was the serum-free floating culture of embryoid body-like aggregates with quick reaggregation (SFEBq) method. This method requires the PSCs to be placed in low attachment substrate; growth factors are used to stimulate cell differentiation and regional specification (Fig. 8.1). The SFEBq method has been used to grow and differentiate specific brain tissues such as substantia nigra, septum, striatum, cerebellum, and dentate gyrus, cerebral cortex, pituitary gland, and retina (Brewer 1995). A critical limitation of this technique is the low survival rate of the tissues in culture (Wataya et al. 2008). Improvements in this technique such as

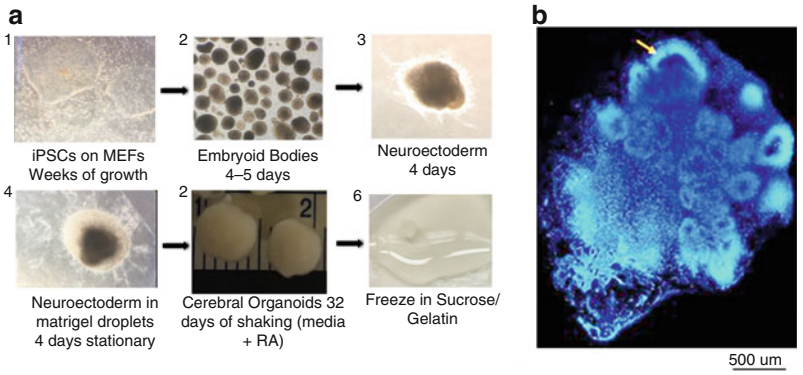

Fig. 8.1 Generation of brain organoid from human iPSCs (Stachowiak et al. 2017). (**a**) Mouse embryonic fibroblasts (MEFs) are used as feeder cells for iPSC culture (panel 1). The iPSCs are grown in a tissue-culture plate to reach confluence. Subsequently, three wells of iPSC are combined into a single well, and, after an additional 24 h, embryoid bodies (Peters et al.) are developed (panel 2). The EBs are maintained in N2/B27 media with dual SMAD inhibitors (which rapidly increases neural differentiation) exchanged daily for 4–5 days. The EBs are transferred into a 24-well low attachment plate, 1 EB per well, and incubated in neural induction medium forming neuroepithelial tissue (neuroectoderm) (panel 3). The neural induction medium is changed daily for 4 days. Beads of matrigel are made by placing a droplet of matrigel onto dented parafilm, the neuroepithelial tissue is placed onto the matrigel droplet, and then the unit is incubated at 37 °C to polymerize. The formed neuroectoderms in matrigel beads (panel 4) are removed from the parafilm and incubated in differentiation medium, without vitamin A, for 4 days without shaking. After 4 days, the growing neuroepithelial beads are referred to as day 1 cerebral organoids (panel 5), are moved to an orbital shaker, and are shaken in differentiation media with all-trans retinoic acid (RA). The differentiation media with RA is exchanged every 3–4 days. The organoids shown here have been grown for 32 days. Subsequently, the brain organoids are fixed in 4% paraformaldehyde and embedded in 10% gelatin/7.5% sucrose (panel 6) and slowly frozen in liquid N_2 to mitigate the cell damage caused through the freezing process. The organoids are stored at −80 °C. (**b**) Section through cerebral organoid—tile scanning of DAPI. A yellow arrow points to cortical rosettes. The organoids formed polarized structures with a distinguishable border that separated a forebrain-like region containing multiple rosettes from a hindbrain-like structure, which typically lacked rosettes (bottom pole), as reported previously (Lancaster and Knoblich 2014). The rosettes contained a ventricle-like lumen surrounded by distinct layers of cells (see Fig. 8.2)

implementation of Rho-associated protein kinase (ROCK) inhibitor to reduce apoptosis and B27-supplemented neurobasal medium optimization have been adapted from other protocols (Brewer et al. 1993).

A critical breakthrough in the generation of cerebral organoids were brain-like aggregates of the ESC-derived neural cells described for the first time by the Yoshiki Sasai laboratory in Japan in 2008 (Eiraku et al. 2008). Later, Juergen A. Knoblich and Madeline Lancaster working at the Institute for Molecular Biotechnology in Vienna (Austria; Lancaster and Knoblich 2014; Lancaster et al. 2013) have published protocols of generating the brain-like organotypic cultures. Their intention was to grow a monolayer of ESCs, but much to their disappointment the explants would not attach to the substrate. ESCs in culture were organized into spherical structures. Madeline Lancaster would eventually call these spherical structures

"cerebral organoids" and others had referred to them as "mini-brains." Nature unveiled in this way an important secret about pluripotency, a secret that was previously passed on to Mina Bissell (Howlett and Bissell 1990) more than 20 years ago, but that for some reason had been forgotten. As we mentioned before, Dr. Bissell discovered organoids in the 1990s (Howlett and Bissell 1990). Yet, it took more than 20 years and the rediscovery of organoids by Yoshiki Sasai and Madeline Lancaster for the scientific community to catch up and to appreciate the magnitude of this breakthrough. The conclusion driven by the research of these two scientists was simple: for organoids, the context matters (Eiraku et al. 2011). The 3D expansion so brilliantly noted by Dr. Bissell and later on by Dr. Lancaster was only possible after extracellular matrix or matrigel was added to the mix (Lancaster et al. 2013; Lowenthal and Gerecht 2016). By using RT-PCR, Lancaster's group was able to identify the formation of different brain regional entities in the organoids such as forebrain (BF1+ and Six3+) and hindbrain (Krox20+, Isl1+). By using immunohistochemistry, they were able to identify dorso-cortical forebrain (Emx1+), ventral forebrain, hippocampus, and choroid plexus, among others (Lowenthal and Gerecht 2016; Lancaster et al. 2013).

The method for growing organoids, as described by Lancaster et al. (Lancaster and Knoblich 2014), consists of two phases: (1) induction of neural identity and differentiation and (2) 3D self-assembly of the early brain structure. Induction of neural identity and differentiation can be achieved by placing the PSCs into ESC medium with ROCK inhibitor. This process leads to the formation of embryoid bodies (Peters et al.), the outer surface of which contains ectoderm, which in turn will give origin to the neural tissue. In the center of the EBs, a mesoderm tissue forms but does not develop. Neural ectoderm is subsequently replated in a differentiation medium containing B27 supplement, 2-mercaptoethanol, and insulin. Later on, phase 1 retinoic acid (RA) is added to the mixture. In the second phase, the neuroectoderms are grown in a simulated low gravity condition for the 3D self-organization to take place.

The developing cerebral organoids displayed apicobasal cortical polarity (12) and for the first time offered a great example of corticogenesis occurring and being observed *in vitro* (Lancaster and Knoblich 2014) and in Fig. 8.1. This new technology opened the early period of human brain development to a direct scientific exploration.

Alongside organoid studies using ESCs, the development of iPSCs brought the organoid technology to the forefront of biomedical research for its potential in observing human disorders. Combining the organoid technology with iPSCs begins to shed light on early human brain development and its perturbation in diverse disorders. It offers a novel ex vivo diagnostic tool and potentially new preventive and corrective treatments that might eradicate the disease. Also, in the transplantation therapy, the risk of immune rejection will be greatly reduced if patients receive tissues that are derived from their own cells (autologous iPSCs).

8.5 Cerebral Organoids from ESCs and iPSCs to Recapitulate Cellular Processes of Cortical Development

Stachowiak laboratory routinely develops organoids using a modified version of the protocol established by Lancaster group. This modified protocol launched in our laboratory is outlined in Fig. 8.1 (Stachowiak et al. 2017). Once the shaking cultures are established, in the following weeks, the organoids gradually increase in size and the number of developed rosettes increases as well. The 21–32 days organoids form polarized structures with a distinguishable border that separates a forebrain-like region containing multiple circular rosettes from a hindbrain-like structure, which typically lacks the rosettes (Fig. 8.1; also Lancaster and Knoblich 2014).

During corticogenesis, neural progenitor cells originate in the ventricular zone (VZ) and proliferate in the subventricular zone (SVZ). Once proliferation ends, the immature, postmitotic neuronal cells migrate outward using established radial glia as a scaffold, with each new round of migrating cells moving past the pervious cell layer formed in an "inside-out" pattern. Distinct layers within the cortex are the intermediate zone (IZ), the cortical plate (CP), and the marginal zone (MZ) (Kriegstein and Noctor 2004): Similar to *in vivo* corticogenesis, organoid cortical rosettes develop three major zones which can be distinguished by nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) and by immunocytochemistry. Figure 8.2b shows stained sections of the organoids developed in our lab using modified protocol (Stachowiak et al. 2017). In DAPI-stained organoid sections (Fig. 8.2a), the VZ contains a ventricle-like lumen surrounded by compact layers of vertically aligned elongated cells. The area outside the VZ, the IZ, contains uniform, predominantly round cells, and the outermost CZ contains horizontally aligned cortical layers. Staining reveals cellular organization consistent with the inside-out pattern of human neocortex development (Kriegstein and Noctor 2004). In addition, proliferative marker Ki67 antigen-expressing NPCs and GFAP-expressing radial glia (Fig. 8.2b, c) are mostly present in the VZ. This is similar to the developing brain in the ventricular and subventricular zones, where the generation of new cells by the brain stem and progenitor cells takes place. Few Ki67-positive cells are also found in the IZ, and proliferating cells are not detected in the CZ. The doublecortin-positive neuroblasts are present in the IZ and the CZ, and βIII-tubulin is expressed by neurogenic radial glia in the VZ and by neuron committed cells (NCCs) in the IZ and young neurons in the CZ (Stachowiak et al. 2017).

8.6 Modeling Human Neurological Disorders

Growing cerebral organoids to the stage where they further replicate tissue organization and neuronal connections and communications provides new means of determining the development and underlying causes of the human neurological

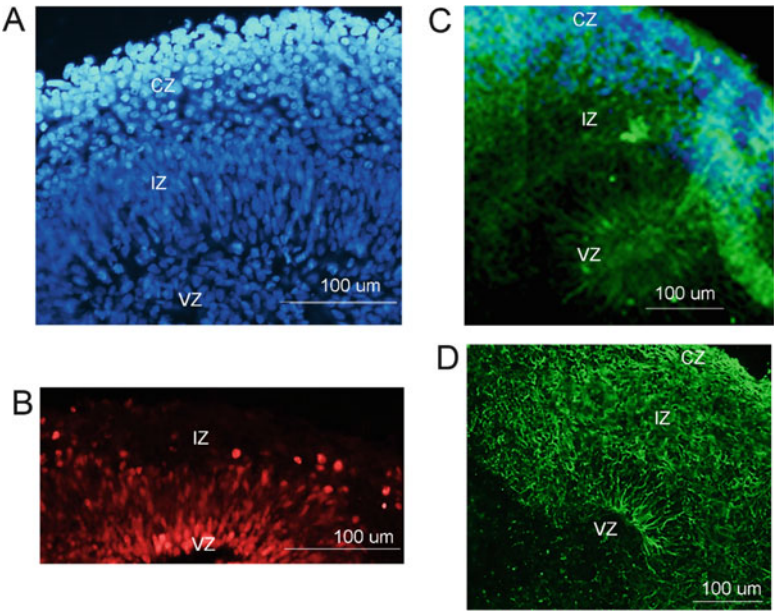

Fig. 8.2 Enlarged view of cortical rosette of the brain organoids (Stachowiak et al. 2017). (**a**) Figure shows three major zones distinguished by DAPI staining. The ventricular zone (VZ) contains a ventricle-like lumen surrounded by compact layers of vertically aligned elongated cells. The area outside the VZ, the intermediate zone (IZ), contains uniform, predominantly round cells. The outermost cortical zone (CZ) contains horizontally aligned cortical layers. (**b**) Ki67 immunostaining shows proliferating neural progenitor cells in the VZ. (**c**) Immunostaining with anti-GFAP antibody reveals GFAP expressing radial glia concentrated in the VZ. (**d**) βIII-tubulin was expressed by neurogenic radial glia in the VZ and by young neurons in the IZ and CZ

disorders. The ability to recapitulate fetal brain development is a critical step to reveal irregular developmental patterns that cause an early developmental disease (i.e., microcephaly and autism), a disease with a delayed developmental onset (i.e., schizophrenia), or leaves the individual with a predisposition to developing a disease later on. Generating organoids from iPSC of the Alzheimer's or Parkinson's patients and healthy subjects and observing changes in tissue structure or protein developmental expression could potentially reveal how certain individuals may be predisposed to the disease later in life and whether this predisposition comes from fetal development (Raja et al. 2016). The organoid model can also be combined with drug therapies to see if certain drugs can reverse or counteract changes in protein expression and enhance proper function (Choi et al. 2016).

The first disease model in human cerebral organoids was genetically based microcephaly. Organoid modeling of microcephaly has been successfully done and studied by the same group of researchers who developed iPSC cerebral organoid protocols (Lancaster et al. 2013). Their studies illustrated successful development of microcephaly from iPSC cultures with a key developmental feature, i.e., premature neuronal differentiation (Lancaster et al. 2013) that could be a cause of the disease.

One of the organoid research areas expected to lead to clinical treatments are studies of the effects of infectious agents and the immune factors on brain development. Brain organoids were used by Patricia P. Garcez et al. to identify the effects of Zika virus (ZIKV) on the ongoing development of the prepubertal brains (Garcez et al. 2016). ZIKV infection being present during gestation, in the placenta and embryo, had only been correlated with the occurrence of microcephaly in the infant. The controlled infection of neural cells and brain organoid permitted a direct examination of the ZIKV-infected developing brain. Human iPSC-derived NSCs were infected with ZIKV and then developed into neurospheres and brain organoids alongside the uninfected cells. The model allowed observing structural changes in developing neurospheres while quantifying the presence of ZIKV in the membranes, mitochondria, and vesicles of the infected cells. Infected neurospheres showed increased apoptosis and reduced neurosphere growth, with cell detachment and smooth membrane structures occurring in the neurosphere infected by ZIKV. When already formed brain organoids were exposed to ZIKV, the investigators found 40% reduction in organoid growth. These results were consistent with the pathological and clinical manifestation of the ZIKV-associated human microcephaly. These findings will likely aid in the development of new treatments of the Zika infections and of other related diseases. Similarly, our team has initiated new cerebral organoid studies to investigate how the immune factors produced during pregnancy may affect early human brain development. Here also, this type of research may lead to new preventive treatments.

8.7 Polygenic Neurodevelopmental Diseases

In schizophrenia and autism spectrum disorder (Stefansson et al.), hundreds of genomic traits have been identified as possible drivers for the disease phenotype (described further below) causing the study of each individual mutation a daunting task (Sanders et al. 2012; He et al. 2013). Hence, some researchers have adopted a different approach for studying these conditions. Instead of trying to understand how each one of these individual mutations can contribute to the general disorganization of the brain function, they are now identifying main pathways affected by these mutations as a whole, in an approach they call the watershed-hypothesis approach (Narla et al. 2017; Cannon and Keller 2006). A similar approach is also being used to study the ASD. Here too, scientists are trying to identify the convergent pathways affected by these mutations (He et al. 2013).

Based on certain clinical and genetic similarities between schizophrenia and ASD, it has been broadly believed that these two conditions might be related (Canitano and Pallagrosi 2017). It has been theorized that at the base of the social and cognitive disturbances, common to both conditions, there is an imbalance between excitatory/inhibitory neuronal activities. This imbalance can be a consequence of disrupted cortical architecture, mainly between the pyramidal glutamatergic neurons and inhibitory GABAergic parvalbumin interneurons. Such

a hypothesis is supported by findings reported by multiple groups. For instance, Zielinski et al. reported an increase in the thickness of the frontal lobe cortex in early childhood and a subsequent arrest in development during late childhood in autistic patients (Donovan and Basson 2017; Zielinski et al. 2014). These findings are supported by a recent study, which used the iPSC-derived brain organoids from patients with autism. The study suggests an increased production of inhibitory neurons caused by increased FOXG1 gene expression (Mariani et al. 2015).

Whether schizophrenia and ASD are related or not, ought to be explored. Yet, if what is causing the misbalance between excitatory and inhibitory neuronal activity is indeed a disarray of the cortical structure, using a mouse model will limit the insights, given the important differences in the cortical microanatomy between species (Quiñones-Hinojosa and Chaichana 2007; DeFelipe et al. 2002; Marin et al. 2001; Anderson et al. 1999; Tan et al. 1998)

8.8 Schizophrenia iPSC Cerebral Organoids

A groundbreaking knowledge that comes from the studies employing iPSC cerebral organoids concerns schizophrenia (Stachowiak et al. 2017). Schizophrenia is the most severe mental illness affecting 1.5% of the world population that has been plaguing the mankind throughout its history. Written references to the schizophrenia-like illness can be traced to the old Pharaonic Egypt, where the thought disturbances typical to schizophrenia, depression, and dementia were regarded as symptoms of an ill mind, which at that time had been synonymous with the heart.

In the Middle Ages and in the Renaissance, mental illness was described as religious-like phenomenon and in later times as a phenomenon of civilization and culture. At some point, the mentally ill were considered to have freely chosen the path of mistake and against reason and morality. The perspective was ethical, not medical, and the treatment included constraints and rewards. This continued until mental illness started to be perceived as a product of natural causalities and an object of medical inquiry. Schizophrenia was classified as a distinct mental disorder, a "dementia praecox" by Kraepelin in 1887 (Jablensky et al. 1993), and the term schizophrenia, a fragmented mind, was coined by Eugen Bleuler, in 1911 (Nuechterlein and Dawson 1984).

On one hand, schizophrenia has been explained as an effect of an external "milieu" and, on the other, as an effect of the physical state of the brain. Even as recent as 1975 defective upbringing by parents was thought to be ultimately responsible for the disease, by affecting functioning of the adolescent brain (Arieti 1975).

8.9 When Does Schizophrenia Begin?

Typically, the onset of symptoms occurs during adolescence, suggesting that the pathology develops in parallel with symptoms, and is precipitated by stress and other environmental influences. Schizophrenia was looked upon as a functional disorder caused by a dysregulation of neuronal communications, a disorder of the communication software. Eliminating bad external influences and retuning neuronal circuits by psychotherapy and/or drugs were considered as viable means to cure schizophrenia. Meanwhile, an alternative idea has emerged that schizophrenia begins in utero and involves a malconstruction of brain circuits (a hardware disorder), which reveals itself later in life as these circuits become fully functional and utilized. It began with the isolated reports of the structural changes in the brain, especially cortex, observed postmortem in adult schizophrenia patients. An improper clustering of neurons in the cortical layers II, III, and V (Arnold et al. 1997) could arise during the first and early second trimester when cortical structure is laid down (Kneeland and Fatemi 2013). These alterations in neuronal numbers or clustering were not due to neurodegeneration, as no neurodegenerative markers are observed in Schizophrenia.

Schizophrenia appears to be specifically a human condition, a disorder of the association cortices, with especially prominent deficiencies in the dorsolateral prefrontal cortex (PFC). True dorsolateral PFC is found only in higher primates, and especially in humans, it is characterized by highly elaborate pyramidal cells with extensive recurrent connections. Schizophrenia is now recognized as an inheritable familial disorder, however one that appears to be the result of interplay between genetic and environmental factors.

While schizophrenia has been shown to be inheritable, the exact genetics behind it is less understood. From over 600 single nucleotide polymorphisms (SNPs), 200 genes and multitudes of copy number variations (CNV) have been found to be significantly associated with schizophrenia (Need et al. 2009; Welter et al. 2014; Malhotra et al. 2011; Kirov et al. 2012; Xu et al. 2008); however, no single alteration makes up more then 1–2% of the schizophrenia population (Xu et al. 2008; International Schizophrenia Consortium 2008; Stefansson et al. 2008). Hence, the genetic causes of schizophrenia appear to be a multiplicity of rare risk alleles and schizophrenia has been defined as a common, rare-variant disease. Also, some environmental factors acting during pregnancy appear to correlate positively with disease incidence (viral infections, nicotinism, etc.)

As mentioned earlier, to explain how various mutations can lead to a common disorder, Cannon and Keller proposed a watershed model (Cannon and Keller 2006) in which individual mutations dysregulate distinct biological pathways, which converge into a common ontogenic pathway(s). The dysregulation of such common pathways was proposed to lead to brain malformations, which increase the risk of the disease. The nature of such pathways has been unknown. Recent genomics-bioinformatics investigation has shown that pan-ontogenic integrative nuclear FGFR1 signaling (INFS) may serve as such pathways (Narla et al. 2017).

8.10 Brain Organoid Study of Schizophrenia (Stachowiak et al. 2017)

It is only now becoming evident, largely due to the organoid-based investigation that schizophrenia indeed entails early developmental malformation of the brain cortex, which is shared by unrelated patients with different genetic backgrounds. More importantly, recent organoid studies carried by our team gave unprecedented retrospective view of schizophrenia. Studies revealed for the first time that the trajectory of the illness was determined at the early stages of human brain development when, its basic structures ventricles, and cortex and the tissue in between are laid down (Stachowiak et al. 2017).

After establishing the protocol for the generation of hESC cerebral organoids, we applied this procedure to human iPSCs lines reprogrammed from schizophrenia and control individuals (Stachowiak et al. 2017), in which common dysregulated transcriptomes have been recently identified (Narla et al. 2017). Below, we describe few of the findings reported in our recent publication. In general, the iPSC cerebral organoids followed the developmental pattern observed in ESC organoids. However, a detailed cellular analysis revealed several significant differences between control and schizophrenia organoids (Stachowiak et al. 2017) (Fig. 8.3). The control iPSC organoids, similar to hESC organoids, contained few layers of NPCs expressing Ki67, a marker protein of the proliferating cells which were restricted largely to the VZ. In contrast, in schizophrenia organoids, the Ki67+ cells were strikingly relocated from the VZ into the IZ, as well as into the CZ (Fig. 8.3a). Computational analyses revealed an increased proliferation and migration of the schizophrenia NPCs in multiple patients' organoids (Stachowiak et al. 2017).

The transcription factor T-Box Brain 1 (TBR1) is expressed by developing neuroblasts, which migrate to and provide the first pioneer neurons of the developing cerebral cortex (Kolk et al. 2006). TBR1 is necessary for neuronal differentiation of NPCs and is a potential master regulator in autism spectrum disorders (Chuang et al. 2015). At 5 weeks of control iPSC organoid development, cells expressing nuclear TBR1 were distributed throughout the entire CZ and IZ (example on Fig. 8.3b). In contrast, in schizophrenia organoids, TBR1+ cells were absent from the upper cortical region, while cells expressing high levels of TBR1 were found concentrated predominantly in deep organoid layers. Consistent with these findings, staining with Pan-Neu Ab which reacts with key somatic, nuclear, dendritic, and axonal proteins of the pan-neuronal architecture revealed differentiated Pan-Neu+ neurons concentrated in the CZ of the control iPSC organoids, forming a distinct cortical layer at 5 weeks (Fig. 8.3). These mature neurons formed a dense network of long processes parallel and perpendicular to the cortical surface. At 5 weeks, the overall density of the Pan-Neu fibers in schizophrenia cortex appeared reduced. This decrease was verified by quantitative measurements (Stachowiak et al. 2017). Instead, the schizophrenia organoids displayed differentiated Pan-Neu+ neurons deep within the IZ and VZ regions (Fig. 8.3d). These mature subcortical neurons were found at 2 weeks in the schizophrenia organoids, at the time when no such neurons were observed in the

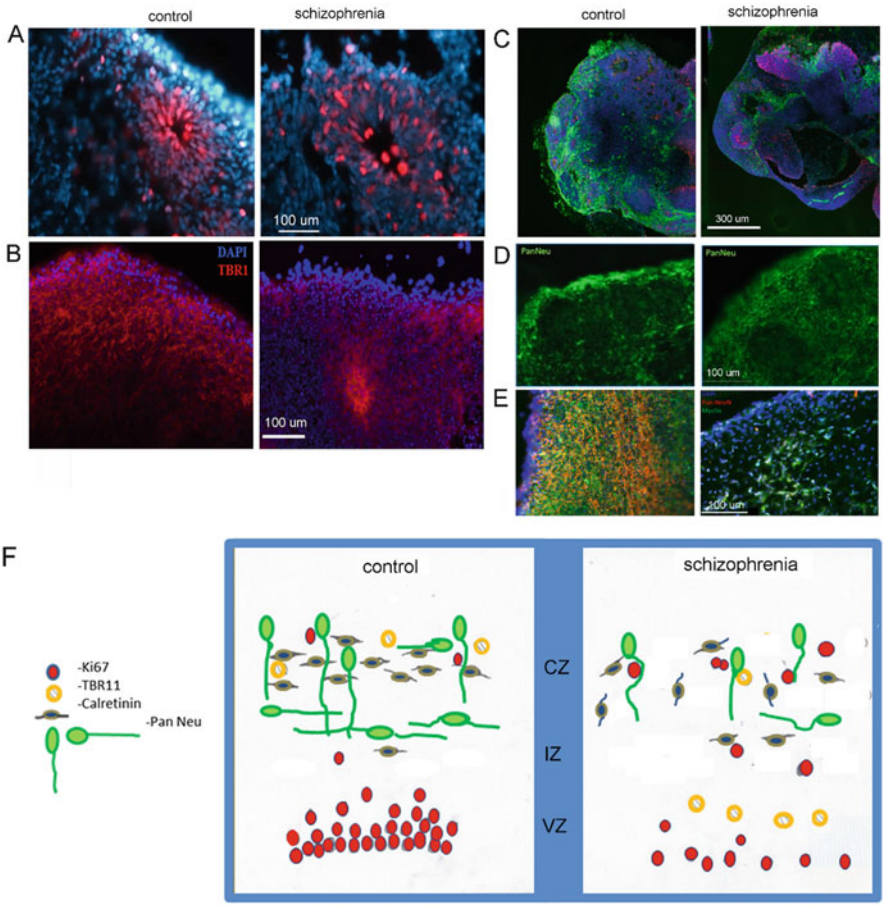

Fig. 8.3 Disorganized migration of proliferating cells and depletion of cortical neurons in schizophrenia 5 weeks iPSC cerebral organoids (Stachowiak et al. 2017). Representative images of control and schizophrenia organoids are shown. (**a**) Organoids were immunostained for Ki67 (red). Nuclei were stained with DAPI (blue) in schizophrenia organoids; note a dispersion of Ki67+ cells into IZ and CZ. (**b**) Decreased nuclear TBR1 (red) expression in the upper cortical zone of 5-week-old schizophrenia organoids. (**c–e**) Reduced density of Pan-Neu+ neurites in basal CZ and the presence of Pan-Neu+ cells with neurites in the IZ are visible. (**e**) Reduction in Pan-Neu-stained neurons (red) and myelinated fibers (green) in the schizophrenia organoid cortex. (**f**) Diagrams show schematic stratification of developing telencephalic-like zones in cerebral organoids—ventricular zone (VZ), intermediate zone (IZ), and cortical zone (CZ). Summary of cortical changes found in iPSC-derived schizophrenia organoids: increased proliferation of Ki67 NPCs and migration from the VZ into the IZ and CZ; reduced cortical accumulation of pioneer TBR1 neurons; reduced formation of Pan-Neu-stained cortical neurons especially those with horizontal neurites; and changes in the orientation of calretinin interneurons (Stachowiak et al. 2017)

control organoids (not shown). Co-staining for Pan-Neu and myelin-associated protein-2 confirmed the reduction neurons and their subcortical projections in the schizophrenia organoids (Fig. 8.3e) (Stachowiak et al. 2017).

Together, our experiments indicate an increased proliferation and migration of the schizophrenia NPCs, a premature development of neurons in the subcortical region, followed by an impaired neuronal development in the cortex of the schizophrenia organoids (Stachowiak et al. 2017). The early changes, increased VZ cell proliferation, migration, and premature neuronal differentiation correlate with the upregulation of gene programs underwriting these functions in early differentiating schizophrenia NPCs (Narla et al. 2017).

An important finding that emerged from this investigation was the role of nuclear form of FGF Receptor 1 (nFGFR1) in the observed dysregulation of cortical development (Fig. 8.3f). At an early stage of the NPC development, nFGFR1 was overexpressed in schizophrenia cells, which appear to correlate with the dispersion of VZ cells and premature formation of neurons in the subcortical tissue. In contrast, at the later stage of cortical development, the nFGFR1 expression was shut off, which correlated with the underdevelopment of the mature cortical neurons and layers. Genomic studies of the NPC and NCCs showed that both an excessive nFGFR1 and diminished nFGFR1 signaling profoundly affected diverse neuro-ontogenic gene programs. Thus, one potential strategy for preventing cortical maldevelopment in schizophrenia could be a normalization of the INFS mechanism.

8.11 "Phase Zero" Clinical Trial Using Organoids

Future applications for iPSC-cerebral organoid-based research in the biomedical field are multifold. They can be broken down into three broad areas of application: (1) organ regeneration and replacement for central nervous system (CNS) injury and degenerative disorders; (2) improved diagnosis of developmental disorders like autism, microcephaly, schizophrenia, and neurodegenerative diseases like Alzheimer or Parkinson disorders; and (3) individualized drug testing and preventive/corrective therapies.

Each of these subject areas has the potential to develop breakthrough discoveries that may not have been predicted or envisioned without the advancement in organoid technology. By sorting through the scientific literature, we can have an idea how proficient basic research is. Yet, translating these discoveries into clinical applications is a costly and long process to say the least (Cerovska et al. 2017). One of the reasons this might be the case is probably the way the drug discovery program is set up. Regulatory agencies require any new drug to be tested first in an animal model or *in vitro* before they can be tested in humans.

Even if the disease may be modeled in mouse, the drug efficacy is often different. So far we have emphasized the important role the genetic profile plays in drug sensitivity testing. For animal and human risk assessments though other aspects need to be taken into consideration. For instance, the response to a drug can vary from one

species to another. Notably, some antibiotics are lethal for hamsters and guinea pigs, yet mice and rats can tolerate them very well. In this sense, we are lucky that Alexander Fleming used rats for testing penicillin, had he tested it on a different rodent, history could have taken a different path.

Aspirin is another excellent example of how a drug can have different effects based on the species. Aspirin is known to cause birth defects in mice and rats but not in humans. Can one imagine what could have happened if aspirin would have been tested on mice? Another such drug known to cause birth defects in mice but not in humans is cortisone. In addition, aspartame is known to cause lymphomas in rats, but not in humans. Acetaminophen, within therapeutic range, is well tolerated by humans but is hepatotoxic in mice at low dose. Indomethacin, a drug used to treat rheumatoid arthritis, is well tolerated in humans; however, it causes ulcers in rats and dogs (Matsubara and Bissell 2016). These examples show that a drug can be safe for rodents, but not for humans and vice versa. No wonder, most of the potential new drugs only ever make it to phase 1 of clinical trials.

The iPSC and organoid technologies bring out a possibility of a new "Phase Zero" clinical trial. Established organoids are grown from control and diseased patients' iPSC in a dish, and the drugs or combination of new drugs is applied to the culture to determine their potential toxic and therapeutic effects. Through this approach, researchers would be able evaluate the drug efficacy against the disease of a specific patient.

8.12 New Directions and Challenges for the Organoid Research

The continued use and development of the brain organoid model will extend into many future scientific endeavors and functional studies. For instance, an abnormal brain development and the responsible agents could potentially be counteracted with the existing and new pharmacological agents. In addition, a promising new tool may be developed to control biological development and functions by incorporating light-activated molecular switch proteins into the cells. One upcoming new development will be partnering of the organoid research with optogenetics and optogenomics. Photonic regulation of light-sensitive switches is highly advantageous compared to classical chemical activation methods, due to its ability to precisely activate and inactivate both in space and in time. Especially the use of the new generation of nano-actuators allows the induction of protein–protein interactions among several proteins of interest on a subcellular scale. Recent advancements in nanotechnology provide the engineering community with a new set of new tools to create nanoscale photonic devices with unprecedented functionalities (Feng et al. 2014; Miao et al. 2016; Nafari and Jornet 2017). In our opinion, the plasmonic nano-lasers working in conjunction with nano-antennas can serve as *nano-photo-actuators* of biological processes. Together, networks of nano-actuators and nano-sensors could control development

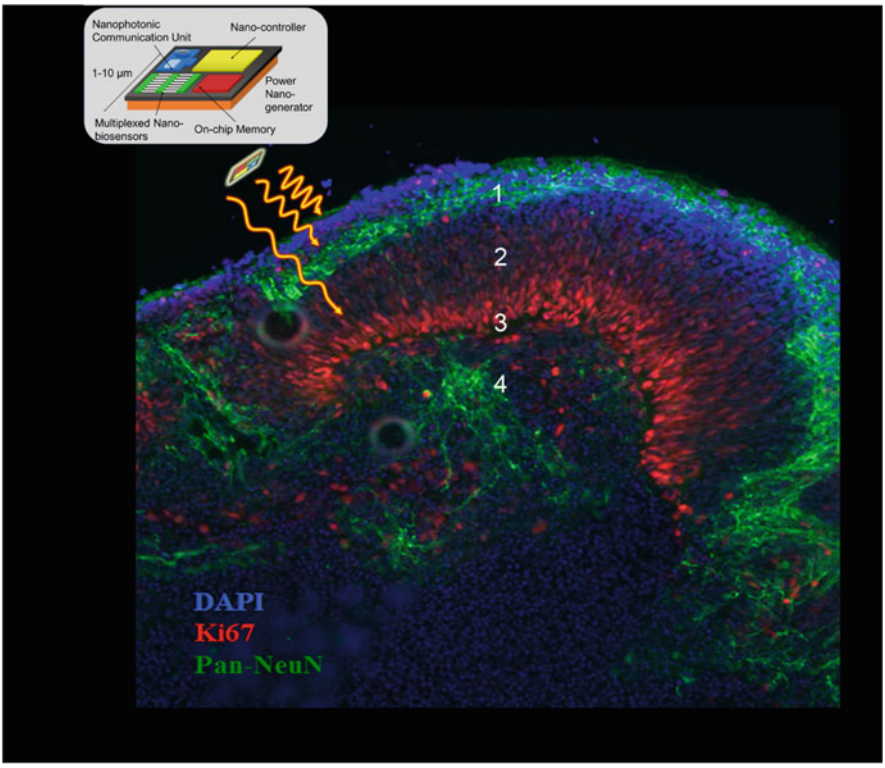

Fig. 8.4 "Chip on the brain"—development of nanophotonic devices for activation and inactivation of molecular switches (protein–protein interactions) to control cell proliferation, migration, and differentiation in brain organoids. Networks of arrayed nano-actuators are developed to control the laser light-sensitive molecular toggle switches. The platform will provide spatial and temporal control of the illuminated area and in addition will contain nanosensors to receive signals generated by the light-emitting cell molecules. The platform diagram was generated by Pedram Johari, the University at Buffalo, Department of Electrical Engineering. The image shows 5-week-old iPSC cerebral organoid: 1—differentiated neurons in CZ; 2—migrating neuroblasts in IZ; 3—proliferating NPC in VZ; 4—neurons in developing deep nuclei

of cells in complex tissues of the developing brain. An important step toward these new technologies will be their testing in the cerebral organoids to see if they can direct and modify organoid development (Fig. 8.4).

It has been proposed that mechanical forces exerted by developing axonal projections influence the surface tension and the shape of the developing human cortex (Hilgetag and Barbas 2006). Cerebral organoids offer an effective tool to test these assumptions and the roles that the changes in surface tension and other vector forces could play in schizophrenia, autism, and other developmental diseases. By combining organoid technology of disease-specific cerebral organoids with the atomic force microscopy (AFM), one could examine for the first time the relationship between tissue elasticity/force and the disease progression (Tan et al. 2017). For instance, in

schizophrenia organoids, changes in cortical axonal tracks, i.e., loss of horizontal and maintenance of vertical fibers, and disorientation of horizontal connecting interneurons suggest that the surface tension and the elasticity of cortex may be reduced (Fig. 8.3f) (Stachowiak et al. 2017). How these altered mechanical properties may affect cortical development and its control by the mechanical forces may be addressed using AFM and a novel optogenomics platform applied to the cerebral organoid model.

Further development of the organoid model faces many technical challenges. If the organoids were to be used for developing new drugs, a better way for delivering drugs and removing wastes ought to be devised. A vascular system is needed to enable the flow of nutrients, oxygen, and hormones in the blood, without which the size of grown cerebral organoids is limited to 5–10 mm and their effective integration with the host tissues may not be possible (Munera and Wells 2017). There are already first reports describing vascularization of organoids generated from iPSCs. In one such study, in 2014, a functional human liver with proper structure was generated from liver buds developed from iPSCs (Takebe et al. 2013). A vascular system within the liver bud transplants began to develop with proper vessel connections (Takebe et al. 2013). This is an important first step in developing regenerative medicine protocols for patients with damaged organs.

8.13 Concluding Remarks

We began this chapter by recounting how generous cancer patients helped in the discovery of stem cells in the adult human brain (Eriksson et al. 1998; Kirschenbaum et al. 1994). It is fair that we come full circle and conclude this chapter by recounting how the latest application of stem cells, the organoid system, is now being used to study cancer. Mina Bissell had studied the murine mammary acinus for a while and is now actively studying breast cancer (Peinado et al. 2017; Snijders et al. 2017). Her research has provided important insights on how the microenvironment, the context as she would call it, can influence the development of cancer (Curtin and Heritier 2017; Cancer Genome Atlas 2012).

In parallel to these cancer-related studies, other researchers are using the organoids to study diverse organ systems including the liver, thyroid gland, pituitary gland, intestines, retina, and the brain (Eiraku et al. 2011; Sato et al. 2009; Antonica et al. 2012; Huch et al. 2013; Suga et al. 2011; Koehler et al. 2013; Xia et al. 2013; Takasato et al. 2014; Taguchi et al. 2014). Combining the organoid technology with iPSCs begins to shed light on how certain human diseases affect early brain development and consequently brain functions. This will likely bring important breakthroughs in understanding the underlying pathologies, improve the diagnostics, and lead to new preventive and corrective treatments. With the cerebral organoids, a frequent common goal has been to expand the cultures in time and develop brain-like structures as advanced as may be possible. Such an approach may potentially be beneficial for regenerative medicine. However, one needs to keep in mind that in

order to identify the role of genome in shaping up the human brain, it is important to focus on the early stages of the organoid development driven by the inherited genomic programs rather than on highly variable advanced stages produced by diverse technical manipulations. Clearly, the advancements in the field of organoids are far from being over.

References

Abdel-Salam GMH, Abdel-Hamid MS, Mohammad SA et al (2017) Aicardi-Goutières syndrome: unusual neuro-radiological manifestations. Metab Brain Dis 32(3):679–683. https://doi.org/10.1007/s11011-017-9993-4

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124(3):319–335

Anderson S, Mione M, Yun K et al (1999) Differential origins of neocortical projection and local circuit neurons: role of Dlx genes in neocortical interneuronogenesis. Cereb Cortex 9 (6):646–654

Antonica F, Kasprzyk DF, Opitz R et al (2012) Generation of functional thyroid from embryonic stem cells. Nature 491(7422):66–71. https://doi.org/10.1038/nature11525

Arieti S (1975) Child and adolescent psychiatry, sociocultural and community psychiatry. In: American Handbook of Psychiatry, vol 2. Basic Books, New York

Arnold SE, Ruscheinsky DD, Han LY (1997) Further evidence of abnormal cytoarchitecture of the entorhinal cortex in schizophrenia using spatial point pattern analyses. Biol Psychiatry 42 (8):639–647

Barker N, van de Wetering M, Clevers H (2008) The intestinal stem cell. Genes Dev 22 (14):1856–1864. https://doi.org/10.1101/gad.1674008

Bartfeld S, Clevers H (2017) Stem cell-derived organoids and their application for medical research and patient treatment. J Mol Med (Berlin, Germany) 95(7):729–738. https://doi.org/10.1007/s00109-017-1531-7

Brewer GJ (1995) Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J Neurosci Res 42(5):674–683. https://doi.org/10.1002/jnr.490420510

Brewer GJ, Torricelli JR, Evege EK et al (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35 (5):567–576. https://doi.org/10.1002/jnr.490350513

Broccoli V, Giannelli SG, Mazzara PG (2014) Modeling physiological and pathological human neurogenesis in the dish. Front Neurosci 8:183. https://doi.org/10.3389/fnins.2014.00183

Cancer Genome Atlas N (2012) Comprehensive molecular characterization of human colon and rectal cancer. Nature 487(7407):330–337. https://doi.org/10.1038/nature11252

Canitano R, Pallagrosi M (2017) Autism spectrum disorders and schizophrenia spectrum disorders: excitation/inhibition imbalance and developmental trajectories. Front Psychiatry 8:69. https://doi.org/10.3389/fpsyt.2017.00069

Cannon TD, Keller MC (2006) Endophenotypes in the genetic analyses of mental disorders. Annu Rev Clin Psychol 2:267–290. https://doi.org/10.1146/annurev.clinpsy.2.022305.095232

Carrel A (1912) Pure cultures of cells. J Exp Med 16(2):165–168

Carrel A, Burrows MT (1911) An addition to the technique of the cultivation of tissues in vitro. J Exp Med 14(3):244–247

Cerovska E, Elsnerova K, Vaclavikova R et al (2017) The role of membrane transporters in ovarian cancer chemoresistance and prognosis. Expert Opin Drug Metab Toxicol 13(7):741–753. https://doi.org/10.1080/17425255.2017.1332179