Chapter 4
Differentiation of Stem Cells into Neuronal Lineage: In Vitro Cell Culture and In Vivo Transplantation in Animal Models

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Abstract Neurological diseases are the major cause of disability and the second major reason of morbidity globally. In the last quarter century, the number of deaths associated with neurological diseases has risen significantly. It will not be surprising that COVID-19 may result in a significant increase of mental burden on mankind, worldwide. Therefore, there is an urgent need to combat this burden, but accessibility to the brain tissue is difficult and brain architecture of complex network of neurons and support cells is daunting. However, with the advent of stem cells, especially the ability to induce somatic cells into induced pluripotent stem cells (iPSCs) it may be possible to investigate brain structure and function in 2D and 3D model, in vivo preparations, called organoids, and such preparations have been used to study blood-brain barrier and other neurological diseases such as Parkinson's disease. Furthermore, with the techniques of molecular genetics and cellular neurobiology it is now possible to reverse neurological disease(s), such as restoration of vision in an aging animal model by reprogramming the methylation pattern of the genome (epigenome), using selected transcription factors. These developments bode well for a paradigm shift in neurological studies and have great potential for diagnosis and therapeutic approaches that were hardly imagined.

Keywords 2D and 3D cultures · Alzheimer's disease · Brain-on-a-chip · CRISPR · Blood-brain barrier · Embryonic stem cells (ESCs) · Endothelial cells ·

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Epigenetics · iPSCs · Neural progenitor cells (NPCs) · Organoids · Hydrogels · Parkinson's disease · Pericytes · Spinal cord injury · Stem cells

Introduction

Nervous system is the most complex organ in the universe, as it is the center of all human activities by mediating signal processing, executing commands and providing an output that ranges from mechanical movements to desire, emotions and, perhaps, the definition of a personality. To study nervous system and its function, it is important to focus that brain function is derived from a neural cellular process that is encoded in the genome of every cellular type that constitutes the nervous system and its supporting cells; thus, genetic components and their expression hold key to decipher brain. Furthermore, there is a huge diversity in neuron types and neural support cells, such as glial cells and astrocytes, and the location of these different cell types within the nervous system adds further to the diversity and complexity to the neural architecture. There is also an important issue of obtaining human brain tissue for performing experiments, which is both practically and ethically very challenging. This has led to the use of animal models, mostly rodents as a source for studying brain developmental studies and as a disease model.

One key technology that may allow a better grasp of neuronal structure and function is the use of stem cells that may be programmed to acquire different neuron types and study the molecular signaling, axonal outgrowth and neural lineage. With the contribution of the Nobel Laureate, Shinya Yamanaka from Japan in developing techniques to reprogram somatic cells by activation of introduced specific transcription factors, it has become possible to generate iPSCs (Takashi & Yamanaka, 2006; Takahashi et al., 2007, González et al., 2011), the induced pluripotent stem cells, with pluripotency that matches the ESCs, the embryonic stem cells. The iPSCs technology allows generating different neural types and tissues that were not easily accessible to obtain from living organisms. Several chapters in this book have described the generation of stem cells and their uses in a wide variety of cell types; herein, we will focus on differentiation of stem cells into neurons, in vitro cell models and their possible in vivo transplantation in live animal models.

The limitations of animal model systems are that the brain structure and development vary greatly between rodents and humans, and rodent models may not display the human neural disease's pathological and functional features. Producing differentiated human neural cells by inducing human pluripotent stem cells (hPSCs), including PSCs, mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs), has now become routine and a simple and cost-effective manner. It has boosted the neuroscience research field, which was purely a fiction only a decade ago (Takashi & Yamanaka, 2006; Takahashi et al., 2007). In addition, as the patient-derived differentiated cell types can now be obtained, induced pluripotent cells can be harnessed to benefit from animal studies and preclinical investigations.

Regenerative therapy has become achievable largely due to the generation of iPSCs and the ability to coaxing them into desired cell lineages using specific transcription and growth factors, allowing pluripotency of human embryonic stem cells (hESCs), to undergo "directed differentiation," by producing large quantities of transplantable somatic cells in vitro when grown in specifically defined culture media supplemented with growth factors. With the capacity of pluripotency, self-renewal and ability to multiply hESCs can be harnessed to generate cells that can be used in transplantation experiments and a variety of cell types that can be coaxed to study human disorders, including behavioral diseases such as Alzheimer's disease and Parkinson's disease.

Differentiation of Stem Cells into Neural Lineage

Neurons have diverse variety by virtue of their position and functional activity; therefore, the neural subtypes have to be specified when developing methods to make stem cells differentiate into neurons. Embryonic tissue was the source for early experiments in attempts to differentiate stem cells into different neurons, but this approach was difficult largely from ethical grounds.

Reprogramming of somatic cells into iPSCs has been achieved by transforming cells with a combination of four transcription factors, namely Oct-3/4, Sox2, Klf4 and c-Myc, also known as the OSKM factors, giving rise to a standard protocol to generate iPSCs, following the seminal discovery in 2006 Shinya Yamanaka and his colleagues (Takahashi & Yamanaka, 2006; Takahashi et al., 2007). These methods have been routinely followed and have been used to obtain iPSCs for various neuron subtypes (González et al., 2011; Zhang, 2013, 2016; Madhavan, 2018; Marton, 2019; Cakir, 2019; Nakatake et al., 2020). However, many of these methods have different problems in the use of small molecules to direct iPSCs in neuronal differentiation, such as variable cell type heterogeneity, poor efficiency, and costly and time-consuming induction protocols for neural differentiation. To overcome these issues, Wang et al. (2018) reported the use of doxycycline inducible transcription factors (TFs) at safe-harbor loci; adapting a two-step method, these cell lines can be induced to differentiate into either lower motor neurons or cortical neurons, in a simple, quicker, scalable and highly efficient manner (Wang et al., 2018; Fernandopulle et al., 2018).

Recently, in a search for transcription factors that may allow induction of diverse cell types though differentiation from human pluripotent stem cells (hPSCs), Ng et al. (2020) reported 290 transcription factors (TFs); of these, 241 TFs were not identified previously, in only four days without changing the external growth factors and biochemical stimuli. Using four of these newly discovered TFs, they were able to reprogram hPSCs into oligodendrocytes, vascular endothelial-like cells, fibroblasts and also neurons that can mimic molecular and functional characteristics of primary

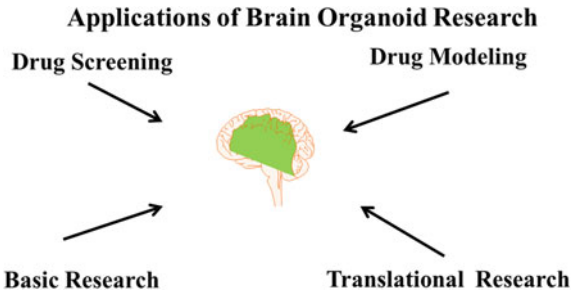

Fig. 4.1 Stem cells in organoid research. From high-throughput drug screening and drug modeling investigations; basic research questions on biochemical and physiological aspects of neural development and axon and support cell growth, etc., and the preclinical translational research; 3D brain organoids have proven their usefulness in multiple ways and elucidated molecular signaling mechanism that were not accessible in cell grown in vitro in two-dimensional format in dishes

cells, enabling programming of hPSCs into different cell types in parallel and simultaneously. The authors further showed generation of cerebral organoids with unmodified hPSCs and oligodendrocyte inducible hPSCs, which enhanced myelination in the 3D brain organoids (Ng et al., 2020) (Fig. 4.1).

In Vitro Tissue Cultures, Transition from 2D to 3D

In most mammals, new neurons are supplied from either the dentate gyrus of the hippocampus, from the cells that are present in this location and are stem cells, or the second source of neurons that is the olfactory bulb, where the neuronal stem cells reside in the lateral ventricle wall, allowing plasticity to the neural architecture and contributing to neurogenesis in adult brain. Haycock (2011) reviewed the 3D cell culture about the current approaches and techniques, arguing that cells traditionally grown in 2D in almost all tissue culture laboratories of the world fail to reproduce the physiological aspects or anatomy of a tissue for a comprehensive study, necessitating the need to develop 3D culture systems, including the consideration of scaffold to support the architecture and organization of the cell assembly or taking into account bioreactors for managing the supply of nutrients and cellular waste disposal. These efforts demand a multidisciplinary approach and expertise to make the 3D culture more appropriate and relevant to physiological recapitulation of the human tissue. Many attributes of neurons make these unique cells, such as the transport of metabolites and factors along the long axons, from the cell bodies to the synaptic region, accomplished by kinesin family of motor proteins on the network of microtubules in each neuron (Siddiqui, 2002). With an increasing sophistication of 3D culture assemblies, it is now possible to co-culture different cell types, including the integration of stem cells and iPSCs.

With the advent of 2D and 3D, organoids and brain-on-a-chip models, investigators have focused on a physiologically compatible model for developmental biology, high-throughput drug screens and preclinical studies, including modeling for neural disorders. Traditionally, neurons have been grown in 2D monolayer tissue cultures similar to other tissue culture protocols, which have been used by researchers to study molecular pathways associated with relatively simple phenomena and allowed basic understanding of neural cell biology, but these 2D monolayers do not represent the complexity of human brain, such as the development and axonal-process outgrowth to form neural networks. This necessity has required developing the 3D brain organoids that could mimic the developmental features and brain architecture in a better way than the 2D monolayer tissue culture.

Among the early research of 3D neural culture system, known as the "neurospheres" assay, this culture system has been used to characterize neural stem cells (NSCs) from the central nervous system (Reynolds & Weiss, 1992). The neural stem cells and neural progenitor cells are co-cultured without an adherent matrix, allowing single cells to multiply to produce small clumps of cells that grow in suspensions and are called "neurospheres," The multi-potent cells comprising these cell clusters can be differentiated into most of the neural subtypes of the CNS, with the exception of neurons and astrocytes (Reynolds & Weiss, 1992). Similar approaches have given rise to the production of "neural aggregates" and "neural rosettes." Neural aggregates are generated from pluripotent stem cells (PSCs), first developing an embryoid body (EB) that is cluster of PSCs recapitulating early embryonic developmental stage. The generated neural progenitor cells are used in monolayer 2D culture of neurons and glial cells by further differentiation of NPCs (Chambers, 2009). Similarly, neural rosettes are composed of neural progenitor cells that may represent the neural tube and show early neural development, and these "rosettes" can be proliferated or differentiated into a variety of mature cells, depicting regional attributes of different regions of the brain (Elkabetz, 2008). Thus, differentiation of pluripotent stem cells (PSCs), such as human embryonic stem cells (hESCs) (Thomson et al., 1998), and induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007) into neural cells in 2D cultures (Zhang et al., 2001, and Chambers et al., 2009) and 3D brain organoids (Fuchs et al., 2004; Eiraku & Sasai, 2012, Lancaster et al., 2013, 2017, Park et al., 2014; Bouyer et al., 2016; Killic et al., 2016, Bordoni et al., 2018) has described experimental models to study central nervous system disorders.

Structure of Brain Organoids and the Gene Expression

Demonstrating the unique self-organizational capacity of human neocorticogenesis, a "cortical sphere" culture system was developed (Kadoshima, 2013). Similarly, the pioneering work of Sergiu Pasca, who developed human cortical spheroids from induced pluripotent cells in a medium lacking the adherent substrate, or the ECM (extra cellular matrix), and with minimal patterning factors to induce cortical spheres, containing both superficial and deep cortical neural cells (Pasca et al., 2015), ushered

a new development in brain organoid cultures. Remarkably, the generated neurons in "cortical spheres" are interspersed with specific astrocyte that is hard to obtain phenotype outside the living brain tissue. These quiescent astrocytes are critical in synaptogenesis and necessary for normal neurodevelopment (Pfrieger & Barres, 1997; Ullian, 2001), and analysis of the transcription showed that 10 weeks of culturing of cortical spheres mimics the transcription pattern of developing human prenatal brain, in vivo. A number of studies have established the 3D brain organoids an important approach to study neural development and disease modeling (Sasai, 2013, Lancaster & Knoblich, 2014; Moreno et al., 2015; Bouyer et al., 2016; Killic et al., 2016; Quadrato et al., 2017; Sartore et al., 2017; Zuhang et al., 2018) (Table 4.1).

Table 4.1 Comparison of in vitro cell culture models; summary of advantages and disadvantages of 2D, 3D and organ-on-a-chip models

Types of in vitro cell culture models	Pros	Cons
2D models Culture plate Transwell membrane	Easy to set up and manipulate High reproducibility Standard and well-established technique Can be set up quickly Low cost	Uniform concentration of nutrients and drugs Not a dynamic state, quite static growth Large quantity of medium, growth factors and drug reagents
3D models Spheroid Organoid Scaffold-type	Recapitulate the 3D Architecture of the cell culture Drug response mimics in vivo concentrations Copies in vivo cell–cell interaction and cell–extracellular matrix interactions Vascularization is possible Blood perfusion is possible	Cellular waste is not removed as in an in vivo model Low reproducibility Poor nutrient transport and access Requires more time to set up Misses the dynamics of in vivo cellular environment, due to the lack of medium fluid flow
Organ-on-a-chip model Microfluidic chip	Diffusion of the medium and drugs is much better, and the microenvironment can be easily manipulated Excellent model for high-throughput screens Actuators and sensors can be easily incorporated and integrated Electronic data acquisition may be possible	Requires external gadgets, such as pumps, valves and circuits to run the experiment Ramping up the culture is difficult Standardization requires extra effort Polydimethylsiloxane, called PDMS used for the fabrication microfluidic chips, and PDMS may adsorb nutrients The microchip setup is costly and requires multidisciplinary expertise to set up

A Comparison of the 2D and 3D Brain Cultures

An important issue in 3D brain organoids is the role of cell–cell interaction that revealed a more complex cell maturation profile in the constructed organoids (Pasca et al., 2015; Kilic et al., 2016; Quadrato et al., 2017; Madhavan et al., 2018; Sloan et al., 2017; Zuhang et al., 2018; Ormel et al., 2018; Qiao et al., 2018). Transcription analysis using RNA-sequencing and whole organoid transcription data has shown similarities in cell composition and transcriptional profiles between human brain cortical organoids and human fetal neocortex (Camp et al., 2015; Kilic et al., 2016; Bouyer et al., 2016; Xiang et al., 2017; Bershteyn et al., 2017). Oftentimes, gene expression levels (mRNA) poorly correlate to expression of specific cellular markers, due to differences in translational profile (Carlyle et al., 2017), that indicated higher amounts of protein expression differences between brain regions compared to the RNA transcription. These differences in the RNA and protein-level abundance suggested functional and cyto-architectural differences between brain regions; e.g., comparison of structurally similar cortical brain tissues showed important differences in abundance between the receptor-associated proteins and resident plasma membrane protein family, which was not evident in the transcription analysis of these tissues (Fig. 4.2).

The basic approach is to select an extracellular matrix material, such as PEG4-MAL (Cruz-Acuna et al., 2017) that can provide the right scaffold for the stem cells to grow and combine appropriate iPSCs in a syringe configuration that may be controlled by microfluidic controls in bio-printing process to develop physiologically relevant brain organoid tissue in 3D.

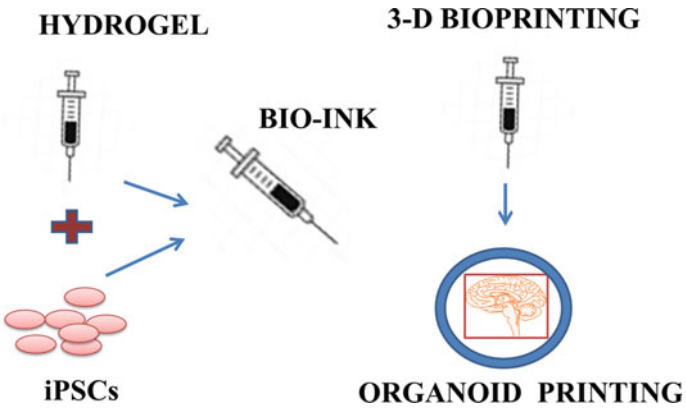

Fig. 4.2 Use of hydrogel and stem cells in bio-printing

Use of Hydrogel and Other Matrix in Brain Organoids

Generation of hPSC-derived human brain organoids has relied upon encapsulation of these brain cell aggregates using Matrigel—produced from biologically derived material—that are poorly characterized and hence show significant lot-based variability and the influence of their biophysical and biochemical attributes, and poor experimental control (Hughes et al., 2010; Miyoshi et al., 2013); Matrigel is obtained from transformed mouse cells and the complexity of these transformed mouse cells precludes its full translational potential (Cruz-Acuna et al., 2016; Gjorevski et al., 2016). Therefore, synthetic hydrogels that are fully defined and can be manipulated for biochemical and physiological properties have great potential alternatives to Matrigel and similar matrices to be used in brain organoid production (Gjorevski et al., 2016), as these could transduce innate cellular behavior by exposure to bioactive motifs, which facilitate cell-directed matrix degradation and cell–matrix-adhesive interactions (Gjorevski et al., 2016; Cruz-Acuna et al., 2017).

Synthesis of PEG-4MAL hydrogel has been described that supports the robust and highly reproducible in vitro generation of human brain organoids from human-induced pluripotent stem cells (hiPSCs) and human embryonic stem cell (hESC)-derived spheroids without the need for Matrigel encapsulation (Cruz-Acuna et al., 2017). Furthermore, PEG-4MAL hydrogel polymerization chemistry allows improved cellular compatibility when compared to free-radical-initiated polymer formation and increased efficiency in cross-linking over acrylate PEG4-A and PEG-4VS (vinylsulfone)-mediated polymerization (Enemchukwu et al., 2016; Cruz-Acuna et al., 2017). The PEG4-MAL hydrogel preparation has an advantage as this hydrogel is not dependent on animal-derived factors such as laminin-111 (Cruz-Acuna et al., 2017), and can be used for both in vitro production and in vivo delivery of organoids, thereby providing an excellent platform for tissue engineering studies and potential therapeutic applications. In addition, mechanical properties of PEG4-MAL synthetic matrix can be manipulated by altering the polymer density, without affecting the hydrogel's adhesive peptide type or density and other biochemical properties (Phelps et al., 2012; Enemchukwu et al., 2016; Cruz-Acuna et al., 2017).

These flexibility features of synthetic hydrogels are important as this allows manipulation of hydrogel properties and can be adapted to promote generation and culturing of a variety of human brain organoids. Hydrogel can also be cost-effective, as it is approximately half the cost of materials when synthetic hydrogels are used as compared to similar amounts of biologically derived Matrigel, which may cost almost twice the cost of synthetic hydrogels (Cruz-Acuna et al.,). In brief, PEG-4 MAL hydrogels can be utilized as in vitro scaffold, which can be manipulated suitable for a variety of developmental requirements, for example, the human brain organoid 3D culture, and not restricted with the limitations of materials that require the preparation from biological sources, such as the Matrigel (Cruz-Acuna et al., 2018).

Increasing the Scalability and Traceability of Organoids

Currently popular techniques to produce mouse pluripotent stem cells-generated organoids are expensive, intensive labor requiring and very difficult to scale, especially by utilizing robotic manipulations. Decembrini et al. (2020) have addressed the issue of scaling up and reproducibility of retinal organoid micro-fabrication, by culturing mouse embryonic stem cells in microenvironments with optimized physical and chemical properties, by using round bottomed milliwells fabricated from biomimetic scaffolds (hydrogels), combined with titrated medium components, resulting in rapid development of retinal organoids from mouse embryonic stem cells in a highly reproducible and efficient manner, such that more than 90% of the cellular aggregates consisted of retinal organoids. These retinal organoids beyond day 26 comprised about 80% of photoreceptor cells, of which about 22% showed GNAT2 marker-positive cone cells that is a critical and rare retinal sensory cell type that is hard to investigate in a mouse model. Thus, the ability to partitioning of retinal organoids into predetermined positions on a 2D array permitted most aggregates into retinal organoids and, furthermore, captured the dynamics of individual organoid that could facilitate for high-throughput screens for drugs and biochemical studies. This protocol combines two key positive developments, to increase the scalability and the ability to trace single retinal organoid, and could permit screens for small molecules that are neuroprotective and a possible source for transplantation of organoids in clinical studies. Decembrini et al. (2020) are credited with an improved technique in that it is based on an a simple one-step handling and manipulation to produce retinal organoids without the need for successive interventions, permitting automation of the 3D culturing process from cell inoculation and seeding to routine medium changes, and also the characterization of retinal organoid growth and differentiation.

Brain-on-a-Chip

The main purpose for developing 3D cell culture systems differs considerably—and ranges from engineering tissues for clinical studies of drug delivery through to the development of drug screening model. The development of the brain-on-a-chip technology primarily has to basically ask which a human brain model can be engineered by cell line assemblies to develop an organ-level model? Hence, it is critical to select appropriate cell lines for such organoids, since brain tissue comprises many different and distinct neuronal types, and additionally a wide variety of supporting cells such as astrocytes and glial cells. The chip design process should also take into account the neural network and brain architecture that varies throughout neural network and brain regions, in a significant and critical manner (Alepee et al., 2014; Park et al., 2014; Bhatia & Ingber, 2014, Jo et al., 2016; Killic et al., 2016; Bouyer et al., 2016; Haring et al., 2017; Qiao et al., 2018, Dolmetsch & Geschwind, 2011; Ducker et al., 2020).

Brain-on-a-chip is a platform, which is engineered to resemble the physiological microenvironment and tissue composition of a specific region of the brain. Therefore, such brain chips have an advantage in their capacity to reconstitute brain microenvironments in vivo, such as cell–cell interaction and scaffold composition, i.e., extracellular matrix and hemodynamics that can be manipulated according to the specific need of the researcher. In contrast, brain organoids investigate the developmental processes to recapitulate the early stages of fetal brain development, such as cell subtype heterogeneity, polarized neuroepithelium and compartmentalization of specific brain regions; furthermore, brain organoid culture has little control over physiological and biochemical factors in the three-dimensional microenvironment, whereas the brain-on-a-chip constructs have limits on the reconstitution of complexity and the temporal and spatial control as seen during the brain development. Thus, to combine the strengths of the brain organoid and the brain-on-a-chip, the organoid on a chip strategy serves as a useful new model synthesizing structural and physiological aspects of the in vivo brain region and the corresponding microenvironment in a 3D space (Park et al., 2014; Moreno et al., 2015; Killic et al., 2016; Skardal et al., 2016, Wang et al., 2018).

Thus in brief, three considerations are important for the brain chip design: first, composition and availability of the cell type; second, the cell maturity; and the third the cyto-architecture, i.e., structural organization of different cell types and their scaffold or matrix for cellular growth within the model. The brain-on-a-chip technique to be functional requires that all required cell types must be available and part of the engineered chip, combining the microsystem platform with hiPSC-derived cell subtypes. Such hiPSC-derived neuronal cell assemblies are useful as these neurons can build a given tissue architectural network depending on their differentiation stage. For a specific application, differentiation can be induced within a specific microenvironment of a compartment, within the physiological constraints of the desired experimental application.

In a brain organoid, in addition to the different varieties of neurons, supporting glial cells such as astrocytes, Schwann cells, oligodendrocytes and microglial cells are also part of the neural tissue, and due consideration has to be made to incorporate the appropriate cell type. These complex multicellular assemblies of brain organoids are necessary for studying the functional nervous system and required for investigating underlying basic developmental processes, including axonal growth and pathfinding, synaptogenesis and neural function. Thus, incorporation of glial and associated cells is critical as these support cells can function as mediators of chemically induced tissue damage and targets of the injury (Alepee et al., 2014). A number of studies have shown that the glial cells could modulate or affect the chemo-toxicity of chemicals for neurons (Schildknecht et al., 2009; Zurich et al., 2002; Vivani et al., 1998), or glial cells may cause neuroinflammatory response of the brain tissue (Falsig et al. 2004; Park et al., 2014; Henn et al., 2011; Boillee et al., 2006, Jasmine et al., 2010, Dolmetsch & Geschwind, 2011, Killic et al., 2016; Bouyer et al., 2016; Jo et al., 2016, Qiao et al., 2018, Wang et al., 2018; Achberger et al., 2019; Ducker et al., 2020). Thus, setting up a 3D brain organoid requires consideration of different types

of glial cells and of course the choice of neuronal cells (Dolmetsch, & Geschwind, 2011).

With the availability of human-induced pluripotent stem cells (hiPSC), the complexity of brain tissue and neural network can be pursued, depending on the differentiation stage of the hiPSCs. The availability of specific stem cells has allowed design of compartmentalized micro-environments within the tissue culture with physiological attributes to attain specific requirements of a specific experimental application. Combining artificial intelligence and bioinformatics with in vitro tissue culture methods can enhance the speed and rate of the drug discovery and drug development process, allow improved pharmacokinetics and toxicological risk assessment and provide better understanding of the neural disease process. Since most of these disease models are organotypic, in which the main purpose is to recapitulate the primary function of an organ such as brain, more than one cell type need to be incorporated in the 3D organoid culture, and the scaffold or the cellular matrix factors should also be considered in the chip design (Fuchs et al., 2004; Morrison & Spradling, 2008, Achberger et al., 2019). Similar approach has been used in evaluation of dental pulp stem cells with different materials to study dental pulp injury (Youssef et al., 2019). It is hoped that the use of such models will increase experimental success by reducing errors and incorrect predictions from small molecule screens for therapeutic development.

Another key consideration in 3D brain organoid technology is formation of cell niches that are specific in the brain developmental process, as they arise by interaction of specific cell types and gradient of signaling factors and stimuli to produce the desirable cellular milieu for the optimum function and development of brain cells. Glial cells are often critical in the formation and modeling of such cellular niches, e.g., niches in the retinal model and niche generated by satellite glial cells in the trigeminal ganglia in association with the pain neuronal cell bodies (Jasmin et al., 2010). Such brain niches and gradients and signaling and trophic factors are required during the neurite growth and guidance and neuronal cell differentiation and thereby in shaping the brain architecture. Thus, such three-dimensional brain organoids are clearly superior to the 2D tissue culture setup when the purpose is to model brain-specific cellular niches (e.g., Fuchs et al., 2004; Morrison & Spradling, 2008, Zuhang et al., 2018; Ducker et al., 2020) (Fig. 4.3).

The architecture includes a flow of medium mimicking the BBB, enriched with soluble factors and peripheral immune cells, which are key players in neuro-inflammation and neuro-degeneration. The migration of peripheral immune cells through the BBB has been implicated in the pathogenesis of several neurodegenerative diseases. The role of infiltrating peripheral immune cells has been investigated in detail for MS, which involves the breakdown of the BBB and multifocal inflammation caused by the innate and adaptive immune systems. However, BBB impairment and the infiltration of peripheral immune cells also correlate with the pathogenesis of other neurodegenerative diseases, such as AD and PD. Adding a fluidic system to mimic the BBB is therefore necessary to investigate the pathological mechanisms of neurodegenerative diseases and eventually to study the transport of drugs across the BBB (Adapted from Slanzi et al., 2020).

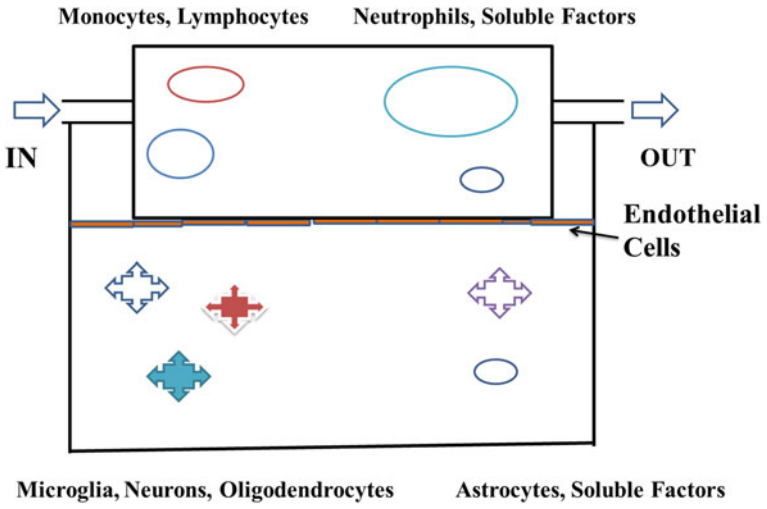

Fig. 4.3 Schematic representation of 3D in vitro models of the nervous system

The third basic requirement for the functional brain organoids is cell maturity, depending on if a organoid is being used to measure toxicity to the mature brain tissue, also known as neurotoxicity assay, or if the experimental design seeks to study perturbations in the development of brain that can be defined as developmental neurotoxicity, thereby requiring different brain organoids to investigate different questions on toxicity or developmental biological studies, respectively (Ducker et al., 2020). Thus, cell maturity in a given model will depend on the type of studies that such toxicity experiments are planned to assess, and these questions have been addressed using, in addition to traditional cell biological features, new technologies such as transcription profiling and the determination of epigenetic state of the cellular genome (Balmer et al., 2012; Waldmann et al., 2014; Qiao et al., 2018).

Stem Cells for Brain Research, HiPSCs from Patients

One alternative to the isolation of neural cells from fetal brain is to generate neural stem cells (NSCs) from pluripotent stem cells, but culturing such cell lines is challenging and requires long periods to generate and propagate; furthermore, the gene expression profile of these cells grown in two-dimensional traditional culture and NSC grown in three-dimensional organoids or chips results in alterations in the gene expression profile, and the cell function also shows distinct differences between the 2D and the 3D neuronal cultures (Koch et al., 2009; Birgersdotter et al., 2005; Zahir & Weaver, 2004).

Since human-induced pluripotent stem cells can generate from somatic cells, brain organoids using hiPSCs can be profitably used in brain organoids and brain-on-chip

technology. One of the main advantages of hiPSCs over primary animal brain or immortalized neuronal cell lines is their constant availability (Takahashi et al., 2007), and that these cells can be differentiated into different neuronal and support cell types. Differentiation of human embryonic stem cells (hESCs), human-induced pluripotent stem cells (hiPSCs) or fetal neural progenitor cells (NPCs) into glial cells and neurons has been reported (Bal-Price et al., 2012; Fritsche et al., 2017). Furthermore, hiPSCs can be derived from human patients and thus can be genetically matched with a desired source (Dolmetsch, & Geschwind, 2011). Human iPSCs (NTERA2) can be differentiated into neural cell aggregates, consisting of astrocytes that interfaced with microvascular endothelial cells derived from human brain, which exhibited the characteristics of blood-brain barrier (Killic et al., 2016). Recently, Goz et al. (2020) have reported such cells derived from glioneuronal tumor, showing that BRAFV600E variants have a cell autonomous effect and the mutation changes several electrophysiological characteristics in neocortical neurons; in contrast, similar neuronal excitability changes were not observed in cells adjacent to BRAFV600E—expressing neurons, showing that BRAFV600E affects a cell autonomous, distinct and highly excitable neuronal electrophysiological response when somatically introduced into neocortical progenitor cells.

Similarly, in another report, Flaherty et al. (2019) showed that hiPSCs generated from NRXN1-alpha the plurality of alternate splicing seen in the human brain tissue, reporting 123 high confidences and in correct reading frame human NRXN1-alpha isoforms. Heterozygous NRXN1-alpha ± hiPSC-neural cells show more than twice inhibition in half of the wild-type NRXTN1-alpha isoforms and transcribe several novel isoforms from the NRXTN1-alpha mutant allele. The authors demonstrated that depending on the genotype, NRXN1-alpha ± mutations can affect the phenotype through the reduction of NRXN1-alpha isoform expression levels and also the presence of the mutant NRXN1-alpha variant isoforms.

In case of familial dysautonomia, where a single mutation can cause a disease, the iPSCs harboring a point mutation in IKBPKAP encoding gene, resulting in the depletion of sensory and autonomic neurons, have been used for wild-type versus diseased hiPSCs screens, for therapeutic drug discovery, and more personal patient-specific diagnosis (Lee et al., 2011, Dolmetsch & Geschwind, 2011). In a brain-on-a-chip variation, Achberger et al. (2019) have used these ideas on retina-on-a-chip (RoC), modeling human retina that combines seven different retinal cell subtypes generated from hiPSCs, demonstrating fluid perfusion similar to vasculature and mimicking in vitro and interaction of mature photoreceptor segments with retinal pigment epithelium (RPE). These authors showed that this interaction supports and increases the creation of outer segment-like networks and recapitulation of in vivo-like biochemical and physiological phenomena such as calcium dynamics and outer segment phagocytosis. This retina-on-a-chip can be used for drug screens such as the antibiotic gentamicin and the retinopathic injury of anti-malaria drug chloroquine, thereby showing promise for drug discovery and a platform to study retinal physiology and pathology of retinal disorders (Achberger et al., 2019).

The Blood-Brain Barrier

As a neurovascular component, the blood-brain barrier (BBB) provides a physical and chemical barrier against intrusion of blood cells, plasma factors and various pathogens for the protection of the central nervous system (CNS). Brain microvascular endothelial cells are the main components of the BBB, together with neurons, astrocytes, pericytes, and the scaffold extracellular matrix (ECM) consisting of type IV collagen, laminin, fibronectin, perlecan and heparin sulfate (Page et al., 2018). Many acute and chronic neural diseases and disorders such as Parkinson's disease, Huntington's disease, ischemic stroke and Alzheimer's disease have been attributed to the malfunction of the BBB (Sweeney et al., 2019).

The blood-brain barrier (BBB) controls the exposure of brain cells in a significant manner; therefore, various in silico or in vitro BBB models should incorporate the choice of cell subtypes, the transport properties and the extracellular matrix to recapitulate the features of human BBB, and a variety of such models has been reported, for example (Vandenhaute et al., 2012). With an increasing sophistication in BBB platform technology (e.g., see Frimat & Luttge, 2019; Hai, et al., 2010; Sweeney et al., 2019; Sun et al., 2020), organoids of different brain regions, such as the cerebral cortex layers, model that were developed by intercalating hydrogel–neuron layers with plain hydrogel layers (Kunze et al., 2011); these cortical layers displayed both the chemical gradient of trophic and growth factors and the differential synaptic density distributed in different layers (Choi et al., 2010).

Pericytes are important for the structure and function of the BBB, and their degeneration is related to neural disorders, with poorly understood mechanisms, due to the paucity of obtaining sufficient pericytes for investigations. Sun et al. (2020) describe pericytes-like cells (PCs) from human pluripotent stem cells (hPSCs) via the intermediate developmental stage of cranial neural crest (CNC) cells and show that CNC-derived pericyte-like cells express specific molecular markers such as NG2, CD146, CD13 and PDGFR-beta, with Vimentin and Caldesmon, and exhibit typical contractile features, endothelial barrier function and potential to form vessels; interestingly implanted into a model transient middle cerebral artery occlusion (tMCAO), with blood-brain barrier disruption hPSC-CNS-PCs are capable of improving functional recovery in the tMCAO mouse model by enhancing the integrity of the BBB and inhibiting neuronal cell death through apoptosis and may provide a model to study BBB function in a variety of neurological disorders (Sun et al., 2020).

Microfluidic engineering has been used to generate BBB models in 3D organoids. In such designs, intersecting channels are separated with a porous membrane (polycarbonate) upon which microvascular endothelial cells and astrocytes (brain) are grown on opposite a section, which in a way recapitulates the BBB and allows for the measurements of trans-endothelial electric resistance (TEER) to evaluate endothelial barrier function (Van Der Helm, 2016). Such BBB models have helped how various drugs and toxins may cross the BBB and find entry to the brain microenvironment.

Another important issue in blood-brain barrier models is to develop innovative drug delivery routes, as the BBB has special requirements for molecular passage

across the barrier. Developing novel drug delivery vehicles is important for drug development of basic physiological studies. Nanotechnology is one of the emerging drug delivery strategies and could have enormous therapeutic potential and translational efficacy; however, there are some problems that remain to be solved, such as the removal of nanoparticles after the drug release and non-specific adverse effects on non-intended tissues and organs, and related toxicity. This will require examining the properties of each nanoparticle design, their intended target and pharmacokinetic properties of this drug delivery (Siddiqui et al., 2020). Thus, developing physiologically relevant models using stem cells can be very useful for drug development, drug delivery and elucidating molecular and structural mechanisms of both acute and chronic neurological disorders.

Neuronal Disorders and Disease Models

A very important use of stem cell technology to mimic brain function is to study neuronal disorders and human neural disease, such as Parkinson's disease and Alzheimer's disease. These disorders affect the integrity of synaptic connections and result in reduction and degradation of these connections, and other ailments such as epilepsy or autism have been attributed to abnormal neural architecture and network responses. For Parkinson's disease (PD) and Alzheimer's disease (AD), brain-on-a-chip approach has been applied and summarized below (Choi et al., 2013; Hai et al., 2010; Lu et al., 2012; Slanzi et al., 2020).

Alzheimer's Disease (AD)

The late onset chronic neurodegenerative Alzheimer's disease is devastating as the dementia grows slowly and develops into irreversible worse outcome over time. Early detection of AD is critical for disease monitoring and management, but conventional methods do not meet these challenges. In addition, animal models that are both expensive and labor- and time-intensive do not allow real-time studies on biological processes underlying the disease, and human and animal species differences also preclude extrapolation of animal studies for the progression of disease in humans. These limitations have prompted investigators to experiment on microfluidic brain-on-a-chip that may mimic the neuroanatomical and physiological features of AD. In Alzheimer's disease (AD), the traditional view is that synaptic abnormalities arise due to accumulation of proteins, such as amyloid-beta and tau protein; hence, some AD studies have focused on production and function of these two proteins on synaptogenesis and communication with the supporting glial cells (Hai et al., 2010); 3D models such as neurospheres have also been employed for AD research (Choi et al., 2010), particularly investigating the amyloid-beta protein expression and synapse formation (Choi et al., 2013). Furthermore, microfluidics technology was used to

show the role of amyloid-beta in synapse formation and in the glia, including the phosphorylation of tau proteins (Cho et al., 2013; Choi et al., 2013; Deleglise et al., 2014; Kunze et al., 2011; Li et al., 2020). In addition, brain-on-a-chip model was also used to show wild-type tau protein transfer across neuron via trans-synaptic pathway (Dujardin et al., 2014).

In another study, fibroblasts from Alzheimer patients who have familial disease (FAD) with mutations in PS1 (A246E) and PS2 (N141I) have been used to generate iPSCs to study neuronal differentiation (Yagi et al., 2011) and showed that FAD-iPSC-generated neurons have higher amyloid-beta42 secretion, mimicking the biochemical pathology of mutant presenilins, and that secretion of amyloid-beta42 from these generated neurons responded well to the gamma secretase modulators and inhibitors, suggesting the possibility of drug screening and validation for high-throughput analysis (Yagi et al., 2011). Thus, such stem cells from hiPSCs derived from patients can provide very useful models to study diagnostic and therapeutic pursuits.

Parkinson's Disease (PD) Model

Parkinson's disease (PD) is progressive neural degeneration disease accompanied by loss of dopaminergic neuronal projections of the ventral forebrain, causing abnormalities in cognitive and motor functions. In spite of considerable efforts in studying PD abnormalities, no drug that can reverse the neurodegenerative process of PD has been discovered (Son et al., 2017; Kouroupi et al., 2020). To investigate mitochondrial transport on single dopaminergic axon, a microfluidic chip of the Parkinson disease (PD) was studied, in which axonal extension was investigated and mitochondria that were labeled were observed (Lu et al., 2012). The chip allowed oriented axonal extension into separate axonal compartments for visualization; in addition, this construction could also allow monitoring microtubule fragmentation and transport of vesicles on microtubules, processes that contribute to the severity of the PD, including the loss of dopaminergic neurons. This provides a great advantage from the conventional 2D culture studies to study the physiological aspects of the PD malformations and other neurodegenerative diseases. In another study, using human neuroepithelial stem cells differentiated into dopaminergic neurons in the microfluidic chip cell culture at a large scale it was shown that this technology could be harnessed to characterize dopaminergic neuron degeneration's marker substantia nigra, which is a specific marker for the progression of Parkinson's disease (Moreno et al., 2015).

Neural Disease Models

A number of other neurological disorders such as amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease), Dravet syndrome, microcephaly, hyper-excitability, epilepsy, autism spectrum disorders and Zika virus-mediated brain malformation are all ready for the use of hiPSCs from the patients in 2D and 3D brain cultures, and organoids and brain-on-a-chip technology for improved understanding of physiological and structural brain studies, and drug screens for a possible therapeutic potential (Morin et al., 2006; Selmer et al., 2009; Gullo et al., 2014; Costamagna et al., 2019; Frimat & Luttge, 2019) (Table 4.2).

Table 4.2 Modeling of neural disease using 3D organoids from human iPSCs

Neural disease	Remarks	Selected references
Alzheimer's disease	Adult onset disease affecting cognition and memory function	Raja et al. (2016) Arber et al. (2017) Gonzalez et al. (2018) Lin et al. (2018) Ranjan et al. (2018) Fan et al. (2019) Ghatak et al. (2019) Meyer et al. (2019) Tzekaki et al. (2019) Choi et al. (2020)
Parkinson's disease	Neurodegenerative disease affecting motor functions, tremor, rigidity and stiffness	Monzel et al. (2017) Ho et al. (2018)
Macrocephaly or the autism	Social interaction disorder, with early onset	Mariani et al., (2015) Ho et al. (2018)
Primary microcephaly	Significantly small head of the newborn, where brain growth is impaired	Kelava et al. (2016) Dang et al. (2016) Lancaster et al. (2013, 2014) Li et al. (2017)
Congenital brain defects and Zika virus-associated malformations	Birth defects in the brain development associated with Zika virus infection	Dang et al. (2016) Cugola et al. (2016) Kelava et al. (2016) Garcez et al. (2016)
SARS-CoV2 virus-associated brain defects	Blood supply to the brain is compromised, with hemorrhage and strokes, loss of taste and smell	Ramani et al. (2020) Song et al. (2020) Mesci et al. (2020) Shpichka et al. (2020) Zimmerling and Chen (2020)
Retinal neuropathy	In diabetes, it is a complication that affects eyes; caused by damage to the blood vessels of the light-sensitive tissue at the back of the eye (retina)	Slembrouck-Brec et al. (2019) Rabesandratana et al. (2020)

Spinal Cord Injury (SCI) and Stem Cell Transplantation

The spinal cord injury (SCI) is a highly common neurological disorder resulting from the destruction of long axis of spinal cord and affects a very large number of young and old people every year, and this is not accessible to simple therapeutic treatments, necessitating combinatory approach to treat SCI, and regeneration of the spinal cord. The SCI results in a cascade of tissue damage, starting with the death of the cells in the central nervous system (CNS), affecting astrocytes, endothelial cells, microglia, oligodendrocytes and, most importantly, neurons. More specifically, long axonal projections are damaged that inhibits descending and ascending axonal pathways that communicate stimuli between the brain and the rest of the body. Subsequently, vascular deterioration causes neuro-inflammation, demyelination, acute injury-associated signaling activation, tissue degeneration and remodeling of the extracellular matrix, enhancing the initial cord injury-associated pathology (Griffin & Bradke, 2020; Hilton & Bradke, 2017; Hilton et al., 2017).

Thus, SCI unfolds a series of physiological and anatomical alterations that can extend from months to years following the injury (Donnelly & Popovich, 2008; Griffin & Bradke, 2020). The key advances required in treating SCI are in nerve regeneration and limiting the tissue damage. For the regeneration of the nerve, tissue engineering and transplantation of appropriate cell subtypes to provide neural protection, axonal growth and path-finding, immune response regulation, myelin regeneration, and neuronal circuitry establishment, to allow a neuron to regenerate and form neural circuitry. The use of induced pluripotent cells (iPSCs) is an emerging technology in treating SCI, and the use of such stem cells also bypasses the ethical problems associated with the embryonic cells or cells from the fetus; thus, neural progenitor cells (NPCs) derived from iPSCs have proven useful after transplantation in animal models of SCI (Nagoshi, & Okano, 2018). One critical bottleneck in using the iPSC-NPCs is the incidence of tumor formation after the cellular transplantation, although some results in marmosets show that iPSC-NPCs mostly differentiated into neural cells around the transplant site, without tumor formation and facilitated axonal regrowth and exhibited vascularization as angiogenesis and protected myelin formation (Griffin & Bradke, 2020; Kobayashi et al., 2012) (Table 4.3).

Transplantation of Stem Cells in Model Metazoans

Therapeutic transplantation application of stem cells, specifically iPSCs, has made great strides. But, it is important to resolve issues concerning immunogenicity and immunological dynamics after transplantation of iPSC-derived cells in such transplantation studies (Itakura et al., 2017). Neural stem cells and neural progenitor cells generated from human and rodent iPSCs (iPSC-NPSCs) can be transplanted in spinal cord injury in animal models (Nori, 2011; Tsuji, 2010), since iPSCs technology allows autologous transplantation. Nevertheless, the limitations are a long waiting

Table 4.3 Spinal cord injury trials—summary of included studies

Trial phase and trial identifier	Name of the trial and the type of cells used in the trial	Intervention and type of the cells used in the trial	Transplantation route
NCT01328860 Phase 1	Autologous stem cells for spinal cord injury (SCI) in children	Autologous bone marrow progenitor cells	Intravenous
NCT01162915 Phase 1	Transfer of bone marrow-derived stem cells for the treatment of spinal cord injury	Autologous bone marrow-derived mesenchymal stem cells	Intrathecal
NCT03308565 Phase 1	Adipose stem cells for traumatic spinal cord injury (CELLTOP)	Autologous, adipose-derived mesenchymal stem cells	Intrathecal
NCT01772810 Phase 1	Safety study of human spinal cord-derived neural stem cell transplantation for the treatment of chronic SCI	Human spinal cord-derived neural stem cell	Intramedullary
NCT03225625 Phase NA	Stem cell spinal cord injury exoskeleton and virtual reality treatment study (SciExVR)	Autologous bone marrow-derived stem cells	Intravenous
NCT02163876 Phase 2	Study of human central nervous system (CNS) stem cell transplantation in cervical spinal cord injury	Human central nervous system stem cell	Intrathecal
NCT03979742 Phase 2	Umbilical cord blood cell transplant into injured spinal cord with lithium carbonate or placebo followed by locomotor training	Umbilical cord blood mononuclear stem cells	Intrathecal
NCT02302157 Phase 1/2a	Dose escalation study of AST-OPC1 in spinal cord injury	Human embryonic stem cell-derived oligodendrocyte progenitor cells	Intramedullary

Adapted from: Platt et al. (2020); Stem Cell Clinical Trials in Spinal Cord Injury: A Brief Review of Studies in the United States, Medicines (Basel) 0.2020;7(5):27

period of several months necessary to induce iPSC to differentiate into the desired mature cell subtype and added cost of scaling-up (Theodorou,). In addition, these autologous iPSC lines require determination of the safety and efficacy of each line; thereby, allogeneic transplantation used in combination with iPSC banks is a better alternative. However, the problem of immune rejection of allograft transplantation

still remains. Cells derived from iPSC show low immunogenicity (Liu et al., 2013), but little is known about immunogenicity or immune rejection of iPSC-generated cells in vivo. One caveat of Itakura et al. (2017) study is that transplantation experiments were done in allogeneic and syngeneic mouse models, which are certainly different from the human immune dynamics. Their data suggest that fetus-NPSCs and iPSC-NPSCs display similar immunogenicity, and that therapeutic cell transplantation into the spinal cord may immunologically better tolerated than transplantation into other organs, which may have some clinical therapeutic potential.

Future Directions

Brain Regeneration: Reversing the Vision Loss in Mouse Model by Reprogramming Stem Cells

A remarkable study published by Lu et al. (2020) reported a huge breakthrough finding in which the authors were able to use genetic reprogramming in old cells to return to their youthful stage and restore vision in a mouse model of glaucoma in aged mice. Since aging gradually degenerates tissue causing cell death and dysfunction and has been postulated to be associated with the epigenetic status of the genome (such as DNA methylation pattern), Lu et al. (2020) investigated whether older animals keep the genetic information needed to restore the epigenetic signature of the young adults, and if so restoring those epigenetic patterns may lead to improvement in the tissue function?

Since there is a gradual loss of function of the central nervous system (CNS) and its capacity to proliferate, Lu et al. (2020) ectopically expressed *Oct4, Sox2, and Klf4 (OSK)* in mouse retinal ganglion cells and demonstrated that transcription pattern and the DNA methylation pattern of the youthful mouse can be restored. Furthermore, they showed that expression of these transcription factors enhanced capacity of axons to regenerate after injury, and the vision loss caused by glaucoma in mouse model can be reversed and vision is restored. The genetic construct used in this study was based on adeno-associated virus (AAV), to allow expression of *Oct4, Sox2* and *Klf4* genes that are expressed in early embryogenesis during early development, and these transcription factors were previously discovered by Shinya Yamanaka's group in Kyoto, Japan (2007, 2012), as the key to induce somatic cells into pluripotent stem cells. The reprogramming of cells by expression of OSK transcription factor to regenerate axons and restore vision was dependent on expression of two DNA demethylases TET1 and TET2. The important improvement in the protocol was to delete the use of c-Myc in the transcription factor cocktail and use only three (*Oct4, Sox2* and *Klf4)* of the four OSKM factors, as no tetratomic growth or cancer was observed in these experiments, as the development of cancerous cells is a huge bottleneck in reprogramming of the iPSCs Fig. 4.4.

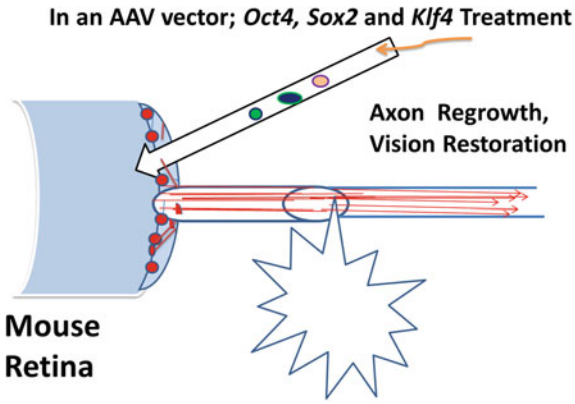

Fig. 4.4 Axonal outgrowth and restoration of vision in a mouse glaucoma model

The retinal ganglion cells (RGCs) communicate visual input from the eye to the brain though axonal connections. Injury to the retinal ganglion axons blocks transmission of this visual information to the brain for processing, causing blindness and loss of vision. Remarkably, Lu et al., 2020, report that damaged retinal ganglion cells (RGCs) can be injected with a cocktail of three transcription factors: *Oct4, Sox2,* and *Klf-4*, also known as OSK factors in an adeno-associated virus (AAV) vector restoring the RGCs to a youthful stage, regrowth of axonal projections and a gain in eyesight. Schematic cartoon is adapted from Lu et al. (2020) and Huberman (2020).

These observations strongly suggest that mouse tissues retain an epigenetic pattern memory of youthful methylation status that pattern may allow designing experiments to facilitate axonal regeneration and improve tissue physiology and function in vivo. The highlights of this important work are that it shows axonal regeneration can be achieved after injury to the optic nerve in mice with injured optic nerves, it restored vision loss in mice with a glaucoma-like condition, and more importantly the technique reversed the loss of vision in older aging animals without glaucoma and in human cells grown in Petri dish. These important observations indicate that aging clocks may be reversed by appropriate transcription control and epigenetic memory recapitulation. The technology is being licensed by Harvard University to a Boston-based company, to try the technique in humans. How the memory of youthful epigenetic state is retained still remains unknown.

CRISPR and iPSC

Another technology that has been by the Nobel Committee and has transformed the biological science landscape is the CRISPR technique and was awarded the Nobel Prize, 2020, in chemistry to Emmanuelle Charpentier and Jennifer Doudna

for discovering one of gene technology's critical tools: the Clustered Regularly Interspersed Palindromic Repeats (CRISPR/Cas9) with Cas9 enzyme, providing genetic scissors for genomic editing (Jinek et al., 2012). It is now possible to use these to change the DNA of animals, plants and microorganisms with extremely high precision. Focusing on neural disease, such as Parkinson's disease, CRISPR technology can potentially allow genome editing, and review of PD patients' iPSCs has been published (Safari et al., 2019; Anzalone et al., 2019; Iarkov et al., 2020). One of the major issues using the CRISPR technology has been the gene alterations in non-specific genome region due to the double-strand breaks in the target DNA or the off-site effect, giving rise to the unintended mutations. However, Rees et al. (2019) have reported a modification in the CRISPR/Cas9 technique in which the Cas9 hybridizes to the target gene site (DNA) using a guide-engineered RNA with a spacer sequence that is complementary; the transfer of this genetic sequence information from these designed guide RNAs helps genomic DNA nicking only in one strand, thereby precluding or greatly reducing the possibility of unwanted DNA nicks in both stands and generation of mutations (Anzalone et al. 2019, 2020). Such approach may revolutionize the therapy of Parkinson's disease and other disorders linked to single-gene mutations.

Conclusions

How do genes control the structure and function of the nervous system is an age-old question that is the key to understand the working of neurons at different levels of complexity and organization (Brenner, 1974; Siddiqui, 1990). New methods and techniques of molecular genetics and cell biology in the last quarter century have given an unprecedented access to the working of brain, such as the discovery of hybridoma technology for generating mono-specific antibodies as a marker for neurons, sequencing of the human genome, labeling cells with green fluorescence protein (GFP) for live imaging of neurons. Similarly, inducing somatic cells to acquire stem cells like pluripotency (iPSCs), including neurons and support cells, using a cocktail of specific transcription factors, the use of CRISPR technology to edit genome at will are great discoveries that promise novel technologies for the mankind. Most recently, the ability to turn the aging clock backward in an old mouse by introduction of OSK transcription factors and reverse the DNA methylation to recapitulate the epigenome of youthful period and in doing so restoring vision in an old mouse and in a mouse with glaucoma and restoration of axonal growth in retinal ganglion cells is a paradigm changing and may be used to reverse not only aging and disease in nervous system, but most likely in other tissues and organs. Stay tuned; there is a lot of good science and discovery that the human brain will continue to contribute, and stem cells and their genetic manipulation will provide new answers to old questions.

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