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A B S T R A C T
Induced pluripotent stem cell (iPSC) technology, which enables the direct analysis of neuronal cells with the same genetic background as patients, has recently garnered significant attention in schizophrenia research. This technology is important because it enables a comprehensive interpretation using mice and human clinical research and cross-species verification. Here I review recent advances in modeling schizophrenia using iPSC technology, alongside the utility of disease mouse models.
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1. Introduction
Schizophrenia is a psychiatric disorder that primarily develops in early to late adolescence, with an incidence of approximately 1 in 100 people (Birnbaum and Weinberger, 2017; Sullivan et al., 2012). In 2011, Japan's medical plan began to include psychiatric disorders, such as schizophrenia, as five major illnesses, which was attributed to an increase in the number of patients and a cause of major social and economic challenges, such as schooling and daily work, the long duration of illness, and substantial suicide-associated risk. Given that some patients are not appropriately treated with the currently available major antipsychotics (Meltzer, 2017; Miyamoto et al., 2005), drug discovery based on molecular pathology of the disease is critical. This would require stratifying the disease based on its underlying molecular pathology and elucidating the pathology of each individual patient group. This in turn would enable a better understanding of the disease, as well as the development of a patient-selective treatment strategy. However, genetic and environmental factors are intricately intertwined in schizophrenia. Accordingly, a definite molecular pathology of the disease has not yet been elucidated (Price et al., 2021).

Recent large-scale genetic studies have identified genetic mutations and genome copy number variants that are likely to be...
strongly associated with the disease (Fromer et al., 2014; Howrigan et al., 2020; Ikeda et al., 2019; Kushner et al., 2018; Singh et al., 2020). Analysis of induced pluripotent stem cell (iPSC)-derived differentiated neurons from patients with such genetic and pathophysiological analysis data will likely facilitate clarifying the molecular pathology of the disease (Balan et al., 2019; Hoffmann et al., 2019; Michael Deans and Brennand, 2021; Nakazawa et al., 2019; Powell et al., 2020; Wen et al., 2016). However, at the cellular level research of iPSCs, it is difficult to confirm the involvement of the molecular mechanisms of the disease. To address this issue, it is critical to perform a multi-scale analysis of neuronal circuits and behavioral levels using a mouse model of the disease by directly introducing genetic mutations as well as performing analysis at the molecular and cellular levels. Furthermore, the validity of the findings obtained from the mouse models should be considered alongside a patient’s clinical features, such as brain imaging data. The use of iPSCs would also facilitate cross-species studies and facilitate a comprehensive interpretation of basic research using mice and clinical research in humans. Currently, the rate of success of drug development in the central nervous system (CNS) is extremely low (Cook et al., 2014). Disease research using iPSC technology will not only clarify the molecular pathology of psychiatric disorders, such as schizophrenia, but will also lead to the development of disease biomarkers, disease stratification based on molecular pathology, and model construction for medication development, which will ensure the success of CNS drug development.

2. Molecular pathological characteristics of schizophrenia

Various studies have proposed numerous hypotheses for the molecular pathology of schizophrenia based on clinical and experimental data (Forrest et al., 2018; Howes and Kapur, 2009; Kid and Kato, 2015; Obi-Nagata et al., 2019; Penzes et al., 2011; Price et al., 2021). Chlorpromazine and haloperidol were introduced into clinical practice in the 1950s. Given the inhibitory effect of antipsychotic drugs on dopamine receptors, the dopamine hypothesis has been proposed. Currently, most antipsychotics have a mechanism of action that inhibits dopamine receptors in the CNS. Furthermore, several pathological hypotheses, such as glutamate, serotonin, gamma-aminobutyric acid, and developmental hypotheses, have been proposed (Laruelle, 2014). The molecular basis of the aforementioned hypotheses includes abnormalities in neuronal cell development, neural circuit formation, synaptic dynamics, and synaptic function. Conventionally, molecular pathological analysis of the disease has been pursued using genetics, animal models, and postmortem brain studies. Postmortem brain research has the advantage of enabling the direct analysis of molecular pathophysiological features, such as abnormalities in epigenetic and transcriptional mechanisms, and post-translational regulation of candidate disease-associated proteins. Despite the challenges associated with the assessment of the validity of model animals, it is necessary to directly validate the possible disease-associated molecular pathology, primarily by neurophysiological and behavioral experiments. In addition, molecular genetics research has been effective in discovering the molecular pathophysiology and, by extension, molecular mechanism-based targets for drug development. In 2014, a genome-wide association study was carried out using a large sample of 36,989 patients and 113,075 healthy subjects and common single nucleotide polymorphisms (SNPs) associated with schizophrenia were identified at 108 loci (Ripke and Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). These included several genes mapped to the gene loci (or their vicinity) related to dopamine, glutamate, calcium channels, and synaptic plasticity. However, the effect size of significant SNPs was low; the one with the highest odds ratio was approximately 1.2. Recently, alongside common SNPs, rare mutations, such as those in GRIA3, XPO7, C11, SETD1A, and GRIN2A, have been identified to be strongly associated with the disease (Singh et al., 2020). Moreover, copy number variants (CNVs), including 3q29 del, 22q11.21 del, 16p11.2 distal del, 7q11.23 dup, 15q13.3 del, and 2p16.3 (NRXN1) del, and de novo mutations that occur for the first time in patients without being present in the parents have garnered growing attention.

3. Studies on the molecular pathological characteristics of schizophrenia using iPSC-related technology

The pathological analysis of schizophrenia has been conducted through clinical and basic research on patients and animal models. However, the inaccessibility of live neuronal cells in patients makes research on schizophrenia difficult, which can be partly substituted by research with disease mouse models that satisfy the construct validity. Nonetheless, it remains necessary to consider the challenge of species differences between humans and mice. Although neuronal types are well conserved in humans and mice (Hodge et al., 2019), there are several genes with different expression levels between species and the presence of several human-specific genes (Suzuki et al., 2018). For these reasons, iPSC-related technology that complements existing research methods has recently gained attention. Since the generation of iPSCs from mice and humans by Yamanaka et al. in 2006 and 2007 (Takahashi et al., 2007; Takahashi and Yamanaka, 2006), researchers have developed neuronal cells derived from patients with schizophrenia and conducted molecular pathological studies. In 2011, patient-derived iPSCs with a mutation in the DISC1 gene were first generated from the fibroblasts of patients with schizophrenia (Chiang et al., 2011). That same year, a detailed functional analysis was performed using iPSC-derived neurons from a patient with schizophrenia (Brennand et al., 2011). Results demonstrated a decrease in neurite length and expression of synaptic proteins, such as PSD-95, and abnormalities in cyclic adenosine monophosphate and Wingless-related integration site (Wnt) pathways. Subsequently, the establishment and analysis of iPSC-derived neurons from patients has been well-documented, as well as abnormalities in the development of neurons, synaptic development, synaptic function, and various intracellular signal transduction mechanisms (Balan et al., 2019; Hoffmann et al., 2019; Michael Deans and Brennand, 2021; Nakazawa et al., 2019; Powell et al., 2020; Wen et al., 2016). As of 2021, approximately 300 papers have been published in the PubMed database on the utilization of iPSC-derived neurons from patients with schizophrenia.

4. Multi-scale studies of schizophrenia using patient iPSC-derived neurons and disease mouse models

One of the advantages of using patient iPSC-derived cells is that it enables the direct analysis of neuronal cells with the same genetic background as the patient. The analysis of iPSC-derived differentiated neurons in patients with disease-associated genetic variants will help clarify the molecular pathology of schizophrenia. However, by cellular-level research on iPSCs, it is currently difficult to demonstrate the involvement of the identified molecular mechanism in disease progression. Accordingly, it is important to conduct neural circuit level and behavioral level analyses, as well as molecular and cellular level analyses. This necessitates the use of a disease mouse model by directly introducing gene mutations occurring in the patient. Furthermore, the validity of findings obtained from disease mouse models should be considered alongside patients’ clinical background, such as neurophysiological and brain image data. Specifically, iPSC technology is important in that it enables a comprehensive interpretation using mice and human clinical research and cross-species verification (Fig. 1).
5. The development of iPSC differentiation technology for pathological research

Despite not being limited to schizophrenia research, it is extremely important to differentiate iPSCs into appropriate cell types for pathological research. The dual Smad inhibition method reported by Chambers et al. (2009) was the fundamental technology for differentiating iPSCs and human embryonic stem cells into various neuronal cells (Chambers et al., 2009). It starts by inhibiting the fate determination of iPSCs to the endoderm and mesoderm by inhibiting bone morphogenetic protein (BMP) and transforming growth factor-β (TGF-β) signals during the initial differentiation of iPSCs, and inducing differentiation into the ectoderm. Subsequently, the ectoderm differentiates into neuronal endoderm and neural stem cells. Currently, there exists a method of culturing in a single layer, similar to a traditionally cultured cell, and a method of forming an embryoid body and a neural rosette (Porterfield, 2020). Differentiated neural stem cells develop into neurons with the application of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF). Consequently, mature neurons generally develop within several months.

In light of the presence of various types of neuronal cells in the human (mouse) brain, cell type-specific differentiation techniques have been actively developed. It is possible to induce differentiation into various types of neuronal cells, such as astrocytes and oligodendrocytes (Fitzgerald et al., 2020; Li and Shi, 2020; McCaughey-Chapman and Connor, 2018). Differentiation techniques for various neuronal cell types that retain regional specificity are based on the manipulation of BMP, Wnt, and sonic hedgehog (SHH) signals involved in the regional specificity of the anterior-posterior and dorsoventral axes of the neural tube. Midbrain dopamine neurons, relevant to schizophrenia, are generated by appropriately activating the Wnt and SHH signals upon induction into neural stem cells, to induce midbrain formation and ventralization (Sundberg et al., 2013, 2021). Furthermore, serotonin neurons are generated from human iPSCs (Lu et al., 2016). Recently, various neuronal cell types have been generated by determining the dorsoventral axis by adding Inhibitor of Wnt Production-2 (IWP-2, an inhibitor of the Wnt pathway), CHIR99021 (a GSK3β inhibitor activating Wnt signaling), and retinoic acid (Imaizumi et al., 2015; Sato et al., 2021). The expression of region-specific transcription factors, such as FOXG1, SIX3, OTX1, OTX2, EN1, HOXB4, HOXC4, PAX6, PAX7, NKX2.1, and NKX2.2, determines the specificity of the differentiated neural cells. However, these methods generally require long-term culture, and further technical improvements may be necessary. In particular, more selective induction methods may be required.

Several methods of differentiation into neuronal cells by overexpressing a transcription factor specific to the nervous system have recently been developed (Oh and Jang, 2019). Furthermore, in 2013, it was reported that almost 100% of iPSCs can be differentiated into excitatory neurons by overexpression of the transcription factor NEUROG2 (or NEUROD1) (Zhang et al., 2013). The latter method is able to assess synaptic function in a relatively short period of time, such as approximately 3 weeks, with high differentiation efficiency. GABAergic neurons can be efficiently produced by expressing miR-9/9* and miR-124 in iPSCs, together with the transcription factors ASCL1, LHX6, and DLX2 (Sun et al., 2016) or by expressing ASCL1 and DLX2 (Yang et al., 2017). In addition, a method for generating dopamine neurons has also been developed (Oh and Jang, 2019). These methods are useful for not only molecular pathological studies but also pharmacological studies since they are relatively easy to scale up.

A number of methods have been reported for producing neuronal cells directly from somatic cells, such as fibroblasts, without undergoing reprogramming (McCaughey-Chapman and Connor, 2018). Examples are that neurons were generated in a relatively short period by expressing the transcription factors (POU5F2/BRN2, ASCL1, and MYT1L) involved in the development of neurons (Pang et al., 2011) or by expressing miR-9/9* and miR-124 together with NEUROD2, ASCL1, and MYT1L (Yoo et al., 2011). These methods primarily use transcription factors and the overexpression of microRNAs (miRNAs), and it is possible to directly generate neuronal cells of patients without producing iPSCs. One of the advantages of direct differentiation is that bypassing the reprogramming step can minimize the destruction of the underlying axis by adding Inhibitor of Wnt Production-2 (IWP-2, an inhibitor of the Wnt pathway), CHIR99021 (a GSK3β inhibitor activating Wnt signaling), and retinoic acid (Imaizumi et al., 2015; Sato et al., 2021). The expression of region-specific transcription factors, such as FOXG1, SIX3, OTX1, OTX2, EN1, HOXB4, HOXC4, PAX6, PAX7, NKX2.1, and NKX2.2, determines the specificity of the differentiated neural cells. However, these methods generally require long-term culture, and further technical improvements may be necessary. In particular, more selective induction methods may be required.
somatic epigenetic profile (Mertens et al., 2015a, 2018). In the case of neurodegenerative diseases, such as Parkinson’s disease for example, neuronal cells produced directly from somatic cells derived from elderly patients are expected to retain the epigenetic profile of old age.

Despite the development of various differentiation techniques, the low maturity of generated neuronal cells is a common obstacle to cellular pathological research, such as investigating synaptic function. Currently, research is being conducted on the maturation of iPSC-derived neuronal cells. One example is that the differentiation and maturation of iPSCs can be achieved by expressing miR-9/9* and miR-124 together with NEUROG2 (Ishikawa et al., 2020). Additionally, although the case with murine iPSCs, the differentiation and maturation of iPSCs can be carried out by adding a mitogen-activated protein kinase kinase (MEK) inhibitor, glycogen synthase kinase 3 β (GSK-3β) inhibitor, and FGFR receptor inhibitor to the medium during the establishment of iPSCs (Nishihara et al., 2019). This result shows that the conditions during reprogramming affect the differentiation ability of established iPSCs. Moreover, 3D-spheroid culture has attracted attention, and the generation of various iPSC-derived 3D-spheroids, including region-specific 3D-spheroids, has been recently reported (Birey et al., 2017; Kadoshima et al., 2013; Lancaster et al., 2013; Pasca et al., 2015; Qian et al., 2016). The 3D-spheroids culture not only reproduces the layered structure of the brain, such as the ventricular and subventricular zones but also matures neurons faster than normal dispersed culture, and is expected to rapidly reproduce the actual state of the patient’s brain (for review, see Qian et al., 2019). Importantly, a method for transplanting human brain spheroids into the adult mouse brain has recently been developed for disease modeling under physiological conditions (Mansour et al., 2018a, 2018b). Given that these technologies have the advantage of avoiding animal experiments, further molecular pathological research using 3D-spheroids will become increasingly important in the future.

6. Studies using patient derived iPSCs with large effect size mutations

In the field of psychiatric disorders, conventional methods, such as genome-wide genetic analysis cannot identify disease-associated genes with large effect size. As a result, the precise molecular pathophysiology of schizophrenia remains unknown. This warrants further studies on common mutations as well as rare mutations with large effect sizes that are supposed directly associated with the disease (Fromer et al., 2014; Howrigan et al., 2020; Ikeda et al., 2019; Kushima et al., 2018; Singh et al., 2020). In particular, despite being rare, research on mutations that lead to a direct understanding of the molecular pathology is critical, as follows: CNV, which is suggested to be strongly associated with the disease, de novo mutations that contribute significantly to the onset of the disease, and disease-specific mutations in patients with schizophrenia from a family with a high disease incidence. On this occasion, clinical information, such as the treatment history and brain function data of the patients, is useful for interpreting the data obtained in studies using patient-derived neurons.

In 2014 and 2015, iPSCs derived from patients with the 15q11.2 del mutation were established (Das et al., 2015; Yoon et al., 2014). The 15q11.2 del region contains CYFIP1, which controls the actin cytoskeleton. Neural cells derived from patient iPSCs demonstrated reduced CYFIP1 expression as well as abnormal cell polarity and dendrite morphology. There are several reports on 22q11.2 del, including abnormal miRNA expression and the increased copy number of retrotransposon LINE-1 in patient iPSC-derived neurons, changes in the expression of miRNA and MAP kinase, and abnormal differentiation into neurons in patient iPSC-derived neurons (Bundo et al., 2014; Toyoshima et al., 2016; Zhao et al., 2015). It is interesting to note that PRKRR-like endoplasmic reticulum kinase (PERK) expression decreases as they relate to the function of the endoplasmic reticulum in midbrain dopamine neurons derived from patient iPSCs as well as abnormalities in the endoplasmic reticulum stress-related system (Arioka et al., 2021). Neurons derived from iPSCs of patients with 15q13.3 del have abnormal transcriptional and epigenetic regulation (Zheng et al., 2021). In addition, patients with 16p11.2 del and 16p11.2 dup and their CNV-introduced iPSC-derived neurons showed abnormalities in neural development, morphology, and synaptic function (Deshpande et al., 2017; Li et al., 2021; Roth et al., 2020; Sundberg et al., 2021). With regard to 2p16.3 del, a deficiency of the NRXX1 gene, there are reports on an abnormality in the release of neurotransmitters in patient iPSC-derived neurons (Avazzadeh et al., 2019; Denault et al., 2019; Lam et al., 2019; Pak et al., 2021). iPSC-derived neural stem cells of patients with 16p13.11 dup show abnormalities in NF-κB signaling and proliferation (Johnstone et al., 2019). For the aforementioned CNVs, disease model mice with a modified region homologous to the human deletion or duplication region have been developed (Hiroi et al., 2013; Hiroi and Yamauchi, 2019; Takumi and Tamada, 2018).

In light of several cases of sporadic onset, de novo mutations that occur in patients (children) with healthy parents are regarded as one of the causes of psychiatric disorders. In recent years, large-scale exome sequencing using samples from healthy parents and patients with autism spectrum disorders has been conducted to identify possible disease-associated genetic mutations at 102 loci (Satterstrom et al., 2020). Functional analyses of various proteins, such as CHD8, SYNGAP1, ADNP, ARID1B, FOXP1, Dyrk1A, and SHANK3 have been reported. However, detailed biological studies focusing on individual mutations are required to elucidate the relationship between mutations in patients and the development of autism spectrum disorders. Recently, iPSCs were established from a patient with a de novo mutation at the POGZ locus (Matsumura et al., 2020). Neural stem cells derived from patient-iPSCs have a lower ability to differentiate into neurons than iPSCs derived from healthy subjects. The molecular mechanism supposedly involves the impairment of the transcription regulatory mechanism by de novo mutation of POGZ (Matsumura et al., 2020). To date, there remains little research focused on disease-associated de novo mutations using iPSCs.

7. Pharmacological studies on schizophrenia using patient-derived iPSCs

Despite many reports on the generation and analysis of iPSCs derived from patients with schizophrenia, there are limited pharmacological studies. Brennand et al. (2011) revealed that loxapine treatment restores the connections between patient-derived neurons and restores gene expression abnormalities (Brennand et al., 2011). Moreover, abnormal protein synthesis in neural stem cells derived from the same patients was improved by the administration of rapamycin (Topol et al., 2015). In 2012, Paulsen Bda et al. showed that valproic acid restored the abnormal production of reactive oxygen species in patient-derived neural stem cells (Paulsen Bda et al., 2012). Furthermore, an analysis of identical twin patients, both with treatment-resistant schizophrenia, was also reported. While one patient was in remission with clozapine, a drug for treatment-resistant schizophrenia, the other did not respond to clozapine. iPSCs were generated from each patient, after which patient-derived neurons were prepared. The treatment of both neurons with clozapine specifically altered the expression of cell adhesion molecules in clozapine-responsive patient-derived neurons (Nakazawa et al., 2017). In 2020, acetyl-l-carnitine was
reported to restore abnormal mitochondrial function in interneurons prepared from iPScs of patients with treatment-resistant schizophrenia (Ni et al., 2020). Furthermore, there are reports of abnormally increased neural activity in iPSC-derived neurons in patients with bipolar disorder. Interestingly, the administration of lithium specifically recovered abnormal neural activity in the iPSC-derived neurons of patients in whom lithium was effective; however, there was no effect in patient-derived neurons in which lithium was ineffective (Mertens et al., 2015b). An increase in inhibitory synaptic density and a decrease in excitatory synaptic density of iPSC-derived neurons were restored by the administration of lithium in an identical twin patient with schizoaffective disorder, bipolar type (Sawada et al., 2020). Other pharmacological studies of patients with autism spectrum disorders (Cavallo et al., 2020; Darville et al., 2016; for review, see Villa et al., 2021), fragile X syndrome (Kaufmann et al., 2015; Kumari et al., 2015; Li et al., 2016; Vershkov et al., 2019), and Alzheimer’s disease (Kondo et al., 2017) have also been reported. Recently, dopamine D2 receptor agonist, which has been used as a symptomatic drug for Parkinson’s disease, has been shown to be effective in inhibiting the progression of amyotrophic lateral sclerosis (ALS) pathology in vitro (Fujimori et al., 2018). Importantly, this study enables stratification of patients with sporadic ALS.

8. Limitations of modeling brain diseases with iPSC technology

Although iPSC technology presents an important solution for psychiatric research, it has several limitations. First, given that iPSC-derived neurons are generally immature even if long period culture, functional analyses of mature synapse and neural circuitry are currently difficult. Transplantation technology may be effective because transplanted human cortical cells exhibit morphological and synaptic maturation in the rodent brain (Espiun-Camacho et al., 2018; Linaro et al., 2019). Second, considering that altered epigenetic profiles are likely to be relevant to brain diseases, complete reprogramming during iPSC generation may be one of the most problematic limitations of iPSC technology. As previously described, bypassing the reprogramming step by direct differentiation can minimize the destruction of the underlying somatic epigenetic profile (Mertens et al., 2015a, 2018). Third, the high heterogeneity of neural cells in iPSC-derived 3D-spheroids or organoids may also be problematic for psychiatric research. Recent advances in single-cell and single-nucleus RNA sequencing technologies have enabled detailed pathophysiological analyses of iPSC-derived neural cells in 3D-spheroids (Lake et al., 2016, 2018; Maynard et al., 2021). Interestingly, single-cell RNA sequencing technologies have identified the molecular pathophysiological mechanisms of schizoaffective disorder, bipolar type in iPSC-derived cerebral spheroids from patients (Sawada et al., 2020).

9. Future perspectives

A phenotypic analysis of genetic mutations and CNVs, which are expected to be strongly associated with disease, would likely enable the stratification of patient groups based on molecular pathology. Conversely, it would also be interesting to stratify patient groups by mathematical analysis using intermediate phenotypes and genomic information of patients and clarify their molecular pathology individually using iPSC technology. In each case, it is critical to elucidate the precise molecular and cellular pathophysiology by multi-scale studies utilizing the neuronal cells derived from the iPSCs of patients, the disease mouse models into which the genetic mutation identified in patients was introduced, and the clinical data of the patient. CNS drug discovery currently suf-fers from an extremely low success rate. In the future, research using iPSC technology will not only clarify the molecular and cellular pathophysiology of schizophrenia but also develop biomarkers for the disease, build model systems for therapeutic drug development, and eventually lead to the success of CNS drug discovery and personalized medicine.

Declaration of Competing Interest

None.

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