Will Brain Cells Derived From Induced Pluripotent Stem Cells or Directly Converted From Somatic Cells (iNs) Be Useful for Schizophrenia Research?

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The reprogramming of nonneuronal somatic cells to induced pluripotent stem cells and their derivation to functional brain cells as well as the related methods for direct conversion of somatic cells to neurons have opened up the possibility of conducting research on cellular disease models from living schizophrenia patients. We review the published literature on schizophrenia that has used this rapidly developing technology, highlighting the need for specific aims and reproducibility. The key issues for consideration for future schizophrenia research in this field are discussed and potential investigations using this technology are put forward for critical assessment by the reader.

Key words: induced pluripotent stem cells/model/brain cells/schizophrenia/experimental design

Introduction

Despite decades of schizophrenia research, only limited etiological progress has been made. A contributing factor is that until recently the only disease-specific human tissue available for study has been postmortem brain. A recent review tabled differences between case and control brains,1 but whether these are disease-induced, compensatory changes, treatment-induced effects, side effects of substance abuse or artifacts caused by numerous, uncontrollable perimortem and postmortem changes is open to debate. Moreover, schizophrenia is widely considered a neurodevelopmental disorder, and so the study of brain tissue collected decades after the primary event(s) may not preserve the critical changes involved in disease onset and progression. Animal models are also challenging to interpret and translate to patients because of species-specific differences between human and rodent brain.2 The recent development of methods for reprogramming nonneuronal somatic cells into induced pluripotent stem cells (iPSCs) and then into brain cells, and of related methods for the direct conversion of somatic cells into neurons (iNs), has created the possibility of conducting research on cellular models from living schizophrenia patients. These models promise to investigate brain cell morphology, electrophysiology, gene expression, and other cellular phenotypes in schizophrenia, leading to new insights into disease biology. Are the available methodologies capable of achieving these goals, or is more work developing the technology and assessing interpatient and intrapatient reproducibility needed before iPSCs or iNS can progress our knowledge of schizophrenia?

What Are iPSCs and iNs?

iPSCs are adult pluripotent stem cells generated from somatic cells by the introduction of a set of transcription factors linked to pluripotency with either the classic virus-based methods3 or other methods developed to avoid integration of foreign DNA into the host genome.4 Brain cells are then derived from the iPSCs by adding a specific combination of growth factors under special culture conditions.5 iNs6 result from the direct conversion of somatic cells into functional neurons or neural progenitor cells (NPCs),7 skipping the stem cell stage. The advantages and disadvantages of both methods need to be considered for specific schizophrenia research aims.

iPSCs in Schizophrenia

Grantees at a National Institutes of Health (NIH) meeting on using stem cells to model psychiatric disorders (October 26, 2010) agreed that the most relevant information would be generated by selecting patients with rare variants of large-effect loci8 rather than choosing heterogeneous cases comprising hundreds of common, small-effect loci9 requiring large sample sizes to generate
meaningful results. Until recently, costs have constrained sample size, but labs are now establishing hundreds of iPSCs (E.W., personal communication).

Since this meeting, several iPSC schizophrenia studies have been published,11–16 some also reporting on neurons generated from these iPSCs.12–14

Proof of Principle

The first publication established proof of principle by generating iPSCs from skin fibroblasts of 2 siblings with a DISC1 mutation (4-bp deletion, exon-intron 12 region).11 Sequence analysis confirmed the mutation in the iPSCs but not in a control cell line. Further work on neurons derived from these iPSCs reported differences in cases and controls similar to those found in the disrupted in schizophrenia (DISC)-knockdown mouse model (Ming, Society for Neuroscience Seminar, 2011). The authors also injected the derived neurons into dentate gyri of severe combined immunodeficiency (SCID) mice and noted integration, proliferation, and differentiation of both case and control neurons for up to 28 days. Recent advances to manipulate transplanted neurons permits the investigation of these cells in preexisting brain circuits.13 iPSCs have also been established from a schizophrenia patient with a 22q11.2 deletion, a rare deletion of moderately large effect.13

Differences Identified Between Cases and Controls

Brennand et al12 studied iPSC-derived neurons from 4 heterogeneous schizophrenia cases and 6 controls. Patient's neurons were observed to have decreased neurite numbers and fewer connections than controls. Of 5 antipsychotics added to the neurons, only loxapine promoted connectivity. The iPSCs were reprogrammed from Coriell cell repository fibroblasts. The 4 cases included 1 male patient with rare, early onset schizophrenia (aged 6) and 3 from multiply affected families including 2 males, 1 with 4 disease-associated copy number variations (CNVs), and their sister. The authors suggested that the observed differences in neuronal conductivity between cases and controls in this small, heterogeneous sample are consistent with the “watershed” model of schizophrenia with multiple genes disrupting key biological pathways. Of 596 differentially expressed genes, 25% had been previously reported in schizophrenia. However, a lentiviral reprogramming method was used, and it has been reported that the genome and epigenome are compromised by such methods.18

A Brazilian group studied cell respiration and oxidative stress response in neural precursor cells derived from 2 iPSC clones from skin fibroblasts of a 48-year-old clozapine-resistant female schizophrenia patient.14 Compared with control clones from an age-matched male, both patient-derived neuroprogenitor lines showed a 2-fold increase in extramitochondrial oxygen consumption and increased levels of reactive oxygen species (ROS). These changes reflected those in schizophrenia postmortem brain and nonneuronal cells. The ROS levels normalized when the mood stabilizer, valproic acid, was added.

Impaired neuronal differentiation and maturation has been reported when iPSCs from hair follicles from 3 clozapine-stable schizophrenia patients were used to derive dopaminergic and glutamatergic neural linages. Mitochondrial respiration and its sensitivity to dopamine-induced inhibition was impaired in the keratinocytes and iPSCs; changes in mitochondrial membrane potential, network structure, and connectivity were also observed in the differentiation process of the dopaminergic and glutamatergic cells.16

These valuable data require replication using standardized methods.

Variability in iPSCs and Derived Neurons

Time in culture is an important consideration in iPSCs and iNs. Pedrosa et al13 identified differences in gene expression in undifferentiated iPSCs, and in day 10 and day 32 derived neurons in both cases and controls. CNVs may also be introduced with increasing passage number. Abyzov et al19 reported positive, but nonsignificant, correlations between CNVs identified in iPSCs and passage number, based on whole genome and transcriptome analysis of 20 human iPSC lines from 7 normal individuals.

Even if cases and controls are cultured identically, iPSCs can harbor somatically derived mutations. Abyzov et al19 observed that an iPSC has on average 2 CNVs not seen in the original fibroblasts because they are consequences of cloning individual cells capturing their mutational history. It is essential that iPSC variability is not confused with the disease phenotype and that cells are genetically characterized preprogramming and postprogramming to iPSCs and after brain cell derivation.

Vitale et al15 also addressed the issue of genetic variability and cell line stability in their lentiviral generation of 18 iPSC lines from fibroblasts of 4 male schizophrenia cases and age-matched, male controls. Eleven lines were considered pluripotent by Maherali and Hochedlinger’s criteria,20 with some showing instability identified by low numbers of cells expressing the pluripotency stem cell marker stage-specific embryonic antigen 4 (SSEA4). Gene expression differences were identified in iPSCs with high and low levels of SSEA4 in the same individual, but clones from high-SSEA4 cell lines showed good correlation in their expression profiles. Pluritest, a matrix containing transcriptional profiles of iPSCs from different laboratories and used to predict pluripotency, indicated that SSEA4-low cell lines were partially reprogrammed.

This evidence suggests that Maherali and Hochedlinger’s20 minimum set of criteria should be supplemented by Pluritest or other databases such as Stemformatics21 (stemformatics.org) to define pluripotency. The use of at least 3 replicate iPSC lines from the 1 patient, the sharing
of data, and the identification of differences shared with other tissues, including postmortem brain, will help discriminate artifacts from schizophrenia-related changes.

Key Issues in the Design of iPSC and iNs Studies in Schizophrenia

Patients and Controls

To date, sample sizes have been small, with mixed patient selection and variable experimental aims and reprogramming methods. Selection of a defined phenotype (eg, clozapine resistance) and/or a known mutation relevant to schizophrenia could identify specific effects associated with the mutation or phenotype in derived brain cells; whereas the selection of heterogeneous cases could identify pathways for future focus. Work is already underway on schizophrenia cases with the 22q11.2 deletion. The use of age and sex-matched controls (22q11.2 cases without schizophrenia) would be preferable, but these constitute a very limited resource and may develop schizophrenia in the future. Genetic rescue, however, would constitute a suitable control.

Normal individuals (sometimes age and sex matched) have been used to generate control iPSCs and derive neurons. A concern for this design, particularly if sample size is small, is that differences may be overwhelmed by variation due to genetic background. Recently developed alternatives for controls in iPSCs/iNs studies enable the removal of a suspected disease mutation from a cell line and its replacement with normal sequence (genetic rescue). Conversely, a single mutant allele can be introduced into an iPSC line to validate potential iPSC disease models. A partial knock-out of neurexin (NRXN1) in iPSCs has been used to investigate the impact of NRXN1 deletions on neurodevelopment. NRXN1 deletions have been associated with a small percentage of schizophrenia cases and other neurodevelopmental disorders.

Gender

iPSC findings relating to the X chromosome could have implications for the use of iPSCs in female schizophrenia patients. Despite only limited reports associated with schizophrenia, the X chromosome is rich in genes involved in brain development. During normal embryonic development, 1 X chromosome is silenced in every female somatic cell, but the selection of either the paternal or maternal chromosome for silencing is random. However, a nonrandom pattern of X chromosome silencing has been observed in female iPSCs, with either the maternal or paternal chromosome being silenced.

Reprogramming and Cell Sources

Work is progressing on the efficient generation of iPSCs from various cell sources using a range of reprogramming methods. Debate continues on the advantages and disadvantages of using viral integration vs episomal or other methods. Viral methods are known to cause genomic and epigenomic aberrations but are more efficient. The use of excisable constructs could combine the high efficiency of integrating viral methods while limiting the potential for genetic changes. Other methods using recombinant protein, miRNA, and modified mRNA have yet to be fully explored. A more efficient nonintegrating reprogramming method uses a modified Sendai virus vector with a temperature-sensitive mutation allowing shut down of viral replication and the clearing of viruses from induced cells. This method together with T-cell activation has been published for deriving iPSCs from 1 ml of whole blood. Having an efficient method that is less invasive than procuring dermal fibroblasts should maximize participant consent. Even less invasive than blood are the cells collected from urine and hair follicles, which have successfully generated iPSCs.

There is also evidence that iPSCs can retain an epigenetic memory of their cells of origin that could potentially confound reprogramming and specific cell derivation outcomes. Reprogramming can erase somatic epigenetic signatures establishing alternative epigenetic marks, but reprogrammed cells do tend to favor the generation of cell types representing the cell of origin. Many years of medication could lead to epigenetic modifications, but this would be addressed with the use of cells from first-episode drug-naive cases.

Human lymphoblastoid cell lines (LCLs) have been reported as a source for iPSCs, which can be differentiated into neurons using a feeder free, episomal reprogramming method. Even though the parental cell lines expressed viral EBNA1 and other Epstein-Barr virus-related elements, these were not detectable in LCL-iPSCs. LCLs harbor artifacts such as point mutations and CNVs, but lines with mutations confirmed in nontransformed cells could provide a source for known high-risk genetic factors such as specific CNVs. Many facilities manage large LCL collections sourced from comprehensively phenotyped schizophrenia patients and matched controls.

Derived Neurons

A reliable disease model requires the identification of a disease-specific cell type. Protocols exist for differentiating predominantly dopaminergic neurons, glutamatergic neurons, gamma-aminobutyric (GABAergic) neurons, oligodendrocytes, astrocytes, interneurons, and motor neurons. Tran et al have recently tabled those relevant to schizophrenia. The methods for identifying and separating cultured neuronal cell types are still not optimal, and better antibodies for fluorescence-activated cell sorting analysis are required.

Pedrosa et al derived primarily glutamatergic neurons from iPSCs from schizophrenia fibroblasts. A subset of
cells also spontaneously developed along the neural progenitor path and some tested positive for TH, a dopaminergic marker, and GADI, a GABAergic marker. Compared with Brennand et al who generated neurons with a posterior identity, this group studied developing forebrain neurons, considered advantageous for a neurocognitive disorder such as schizophrenia. Gene expression profiling assessed the molecular changes occurring during neuronal generation and also demonstrated expression of many genes of interest in schizophrenia neurobiology. These data have been deposited in Gene Expression Omnibus (accession number GGSE26629).

Direct Conversion to Neurons

Direct conversion of mouse fibroblasts into functional neurons, skipping the stem cell stage, was first published in 2010. This was followed by several reports (reviewed by Tran et al) of direct conversion of human fibroblasts into neurons. Direct conversion is more efficient, simpler, and faster, and it avoids the generation of potential tumorigenic stem cells. However, long-term stability of induced human neurons has not yet been demonstrated. A major disadvantage for schizophrenia research is that this method does not mimic neuronal development. Brennand and Gage commented in the Schizophrenia Research Forum (July 27, 2011): “We worry that bypassing neuronal differentiation and maturation will shortcut the cellular phenotype of neurodevelopmental disorders.” The recently reported technique for a 12-day generation of integration free NPCs from human urine cells could potentially overcome this even though they are more specific than stem cells and unable to divide as many times. These cells were able to generate various subtypes of excitable neurons. Functional in vivo analysis is still to be done and more work is needed to determine the relative ages of any precursor, precursor-derived, or iPSC-derived cells in relation to brain maturation stages.

Possible Investigations Using iPSC-Derived Brain Cells or iNs for Schizophrenia Modeling for Critical Consideration

When reproducible methods and standardized protocols are established, various hypotheses (neurodevelopmental, dopamine, glutamate, N-Methyl-D Aspartate Glutamate receptor (NMDAR) hypofunction, and autoimmune hypotheses) and the genetic influences on the etiology of schizophrenia could be investigated. Cells generated from cases with a known mutation could be used to link the mutation to changes in cellular properties such as morphology, myelination, electrophysiological characteristics, and cell secretions. Glutamatergic, gamma-aminobutyric, and dopaminergic neurons and other brain cell types (e.g., oligodendrocytes, astrocytes, and interneurons) can now be derived representing different brain regions and in vitro investigations of these neurons and their interaction with each other or with other brain cell types could be undertaken. One example is the coculturing of neurons with astrocytes to determine the effect of glial support cells on neurons; another would be the study of interneurons and pyramidal neurons to further investigate the reported role of cortical parvalbumin interneurons in the etiology of schizophrenia. Results (eventually of circuits of iPSC neuron-derived cocultures) could be compared with those from postmortem brain tissue or relevant animal models. DISC1-knockout mouse models and DISC1-mutation-iPSC-generated neurons are already established, and comparison of these is underway (Ming, Society for Neuroscience Seminar, 2011).

Comparing the function of derived brain cells from discordant monozygotic twins may help identify non-hereditary or epigenetic factors and gene expression level changes by using living brain tissue as opposed to post-mortem brain or nonneuronal tissue. The interaction between characterized cells and hormones or chemicals could also be studied. Additionally, immune hypotheses of schizophrenia could be investigated by examining immune dysfunctions, such as imbalances in inflammatory cytokines in cultured cells when challenged by autologous immune cells. Derived specific or nonspecific brain cells or progenitor cells could be used to study a range of autoantibodies in the serum of autologous/non-autologous cases. This broad focus could be narrowed to identify specific antibodies such as the NMDAR subunit autoantibodies recently reported in a relatively high proportion of first-episode schizophrenia patients (10% compared with 0.4% in controls).

Derived brain cells alone may not be appropriate for modeling diseases believed to be outcomes of aberrant neural circuitry where a whole-brain environment is needed to reflect pathology. This could, however, be investigated by injection of derived neurons into the brains of SCID mice or by using the potentially powerful approach of a 3 dimensional (3D) culture involving self-organizing cell aggregates to mimic features of the developing forebrain providing a physiologically relevant assay for altered developmental processes.

Conclusions

Schizophrenia research using iPSCs or iNs technology is very much in its infancy and in need of well-designed, defined experimental aims and methods and reproducible results. The challenges involved and future directions for neurological diseases were discussed comprehensively in a workshop held by the National Institute of Mental Health and the Foundation for NIH in 2012.

Figure 1 summarizes the issues for consideration in an iPSC/iNs project. For iPSC and iNs technology to
Fig. 1. Considerations for specific experimental design.
advance the understanding of schizophrenia, further investigation of the effects of reprogramming and differentiation on the genetics and function of the derived cells compared with natural brain cells is needed. These basic investigations and those relating to understanding the mechanisms of reprogramming are progressing. Once standardized, this technology should enhance the knowledge gained from other schizophrenia research domains.

One of the driving forces behind iPSC development and the generation of specific cell types has been for use in regenerative medicine. However, in the absence of an established causative cell type or disease process, it is difficult to envisage a cellular therapy for schizophrenia at this time. Nevertheless, iPSC technology may well facilitate systematic evaluation of brain cell interactions in 3D culture systems. This may in turn uncover phenotypes that may model schizophrenia more appropriately and/or uncover disease subtypes. Moreover, screening of novel therapeutic agents for schizophrenia on neuronal cell cultures or cultures containing combined cell types may in fact help elucidate their mode of action and help tailor medication regimes to particular subsets of patients with schizophrenia.

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