Modeling Schizophrenia Using Induced Pluripotent Stem Cell–Derived and Fibroblast-Induced Neurons

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Although schizophrenia affects a number of brain regions and produces a range of clinical symptoms, we believe its origins lie at the level of single neurons and simple networks. Owing to this, as well as to its high degree of heritability, we hypothesize that schizophrenia is amenable to cell-based studies in vitro. Using induced pluripotent stem cell–derived neurons and/or fibroblast-induced neurons, a limitless quantity of live human neurons can now be generated from patient skin biopsies. We predict that cell-based studies will ultimately contribute to our understanding of the molecular and cellular underpinnings of this debilitating disorder.

Key words: neurons/stem cells/genetics

Reprogramming of hiPSCs From Fibroblasts

The ability to reprogram patient somatic cells into human-induced pluripotent stem cells (hiPSCs) provides a limitless source of live human cells for modeling schizophrenia. Fibroblasts can be reprogrammed by overexpressing 4 critical genes, OCT4, SOX2, KLF4, and c-MYC.1,2 (c-MYC, a potent oncogene, can be eliminated from the reprogramming cocktail, although this results in a markedly lower efficiency.3) In addition, a second combination of genes, OCT4, SOX2, NANOG, and LIN28, is also capable of reprogramming.4 These reprogramming genes can be overexpressed through a variety of means to be discussed later in this review, including viral transduction or transfection of plasmid DNA or messenger RNA. hiPSCs are comparable to human embryonic stem cells in terms of gene expression, epigenetics, telomerase activity, proliferation, and pluripotency.1,2

Early methods of reprogramming relied upon constitutive retroviral or lentiviral expression systems, with 2 potential limitations: incomplete viral silencing of reprogramming factors and insertional mutagenesis upon viral integration. To address the first, a doxycycline-inducible lentiviral system was developed to repress viral genes after the reprogramming process was complete.5 Although these first-generation methods were ultimately sufficient to generate cell-based models for psychiatric disease,6,7 in the meanwhile, integration-free methods were developed. Adenovirus can generate integration-free iPSCs, but at poor efficiencies, owing to low expression of adenov-receptors in nonliver tissue.8 Plasmid vectors also yield iPSCs at low efficiencies and furthermore require repeated transfections.9,10 Synthetic modified mRNA reprogramming is efficient but requires daily transfections in addition to a proprietary media formulation during the reprogramming process.11 Most recently, 2 groups have demonstrated efficient reprogramming using nonintegrating Sendai virus.12,13 mRNA and Sendai viral reprogramming represent the best and the most robust methods available to date. Kits for both methods are commercially available at comparable costs through StemGent and Life Technologies, respectively. Integration-free reprogramming is now straightforward, although at present, only Sendai reprogramming is effective for both human fibroblast and blood cells (table 1).

Differentiation of hiPSCs to Neurons

Even state-of-the-art hiPSC neural differentiation protocols produce heterogeneous neural populations of mixed spatial and temporal identities. Although the relative frequency of a specific neuronal cell type might be favored, differentiated populations are typically comprised of several types of neurons, as well as astrocytes, oligodendrocytes, neural precursors, and even non-neural cells. Published methods have demonstrated that stem cells can be biased to differentiate toward regional identities including forebrain,17–19, midbrain/hindbrain,20,21 and spinal cord.22,23 The precise temporal age of hiPSC-derived neurons, relative to fetal and adult human tissues, remains unresolved.
Strong evidence now links schizophrenia to aberrant activity of 3 neural populations: cortical glutamatergic neurons, gamma-aminobutyric acid (GABA)ergic neurons, and midbrain dopaminergic (mDA) neurons. Pharmacological and animal studies suggest that disease results, at least in part, due to reduced glutamatergic input onto GABAergic neurons. Conversely, antagonists of dopamine receptors reduce the positive symptoms of schizophrenia, which is also associated with increased dopamine synthesis, release, receptor numbers, and resting-state synaptic concentrations. Both cortical glutamatergic and GABAergic neuronal populations and mDA neuronal populations can now be efficiently differentiated in vitro from hiPSCs. Two groups have recently published methods to differentiate pluripotent stem cells to cortical neurons. One group reported that when neural induction occurs in the presence of 2 inhibitors of SMAD signaling, Noggin, and SB431542, the addition of vitamin A efficiently induces a cortical progenitor population at an efficiency that approaches 100%, which can be further expanded in the presence of FGF2 and differentiated to functional cortical neurons following an extended period of corticogenesis. Consistent with this, the other group showed that induction of forebrain fate can occur in the presence of FGF2 and inhibitors of the bone morphogenetic protein, Wnt/β-catenin, and TGF-β/activin/nodal pathways; further differentiation resulted in populations of glutamatergic and GABAergic neurons expressing cortical markers, albeit at lower efficiencies than the first report, perhaps owing to a lack of retinoid signaling. mDA neurons are particularly relevant to the study of schizophrenia, and efficient protocols have been developed to differentiate pluripotent stem cells to mDA neurons by recapitulating the well-understood program of specification that occurs during embryonic development. Yields have been systematically optimized for the past 4 years, from ~20% to >80% mDA neurons, using a 2-step process. First, neural induction occurs in the context of dual SMAD inhibition. Second, mDA specification occurs following strong activation of SHH signaling (in combination with purmorphamine, a small-molecule SHH agonist) and WNT signaling (via CHIR99021, a potent GSK3B inhibitor). Cell surface markers to determine the exact temporal and regional identity of any individual neuron in a heterogeneous hiPSC-derived neural population are critically lacking; in live cultures, one cannot distinguish whether a neuron is glutamatergic, GABAergic, or dopaminergic (table 2).

### Table 1. Summary of Methods of Reprogramming to iPSCs

<table>
<thead>
<tr>
<th>Species</th>
<th>Factors Involved</th>
<th>Reprogramming Method</th>
<th>Integrating?</th>
<th>Efficient?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse, human</td>
<td>OCT4, SOX2, KLF4, (c-MYC) or OCT4, SOX2, NANOG, LIN28</td>
<td>Retroviral or lentiviral</td>
<td>Yes</td>
<td>Yes</td>
<td>Takahashi et al., 2014; Yu et al., 2014; Park et al., 2015</td>
</tr>
<tr>
<td>Mouse, human</td>
<td>OCT4, SOX2, KLF4, (c-MYC)</td>
<td>Inducible-lentiviral</td>
<td>Yes</td>
<td>Yes</td>
<td>Maherali et al., 2015</td>
</tr>
<tr>
<td>Mouse</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
<td>Adenovirus</td>
<td>No</td>
<td>No</td>
<td>Stadfield et al., 2010</td>
</tr>
<tr>
<td>Mouse, human</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
<td>Plasmid transfection</td>
<td>No</td>
<td>No</td>
<td>Okita et al., 2012, 2016</td>
</tr>
<tr>
<td>Human</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
<td>Plasmid transfection</td>
<td>No</td>
<td>Yes</td>
<td>Warren et al., 2010</td>
</tr>
<tr>
<td>Mouse, human</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
<td>Sendai virus</td>
<td>No</td>
<td>Yes</td>
<td>Nishimura et al., 2012; Ban et al., 2013</td>
</tr>
</tbody>
</table>

### Table 2. Summary of Methods of hiPSC Neural Differentiation Relevant to Schizophrenia

<table>
<thead>
<tr>
<th>Species</th>
<th>Intended Cell Type</th>
<th>Purity</th>
<th>Method</th>
<th>Neuronal Characterization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Cortical glutamatergic and GABAergic neurons</td>
<td>Approaches 100% mixed cortical cells</td>
<td>Dual SMAD inhibition in the context of retinoids, followed by FGF2 treatment</td>
<td>Immunohistochemistry, gene expression, electrophysiology, and transplantation</td>
<td>Shi et al., 2019; Mariani et al., 2012</td>
</tr>
<tr>
<td>Human</td>
<td>Midbrain dopaminergic neurons</td>
<td>&gt;80%</td>
<td>Dual SMAD inhibition followed by SHH and WNT stimulation</td>
<td>Immunohistochemistry, gene expression, electrophysiology, and transplantation</td>
<td>Chambers et al., 2009; Kriks et al., 2011</td>
</tr>
</tbody>
</table>

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Direct Induction of iNeurons From Fibroblasts

Following the demonstration that a terminally differentiated cell could be reprogrammed to a pluripotent state, the next question was whether fully mature cells could be directly converted from one fate to another. Mouse fibroblasts were converted into induced neurons (iNs) by overexpressing Brn2, Ascl1, and Myt1.32 This finding was replicated in human fibroblasts, using the same 3 factors—BRN2, ASCL1, and MYT1—with the addition of NEUROD1.33 The conversion takes as little as 6 days and persists after the silencing of viral expression, but is inefficient, occurring in just 2%–4% of the original fibroblasts. Notably, these iNs, although capable of induced action potentials, were relatively immature and unable to form synapses without the presence of mouse cortical neurons.

Independently, 2 groups demonstrated that the addition of key neuronal microRNAs to the induction yielded iNs with functional synapses in pure cultures. The addition of microRNA-124 to the factors MYT1 and BRN2,34 or the addition of microRNA-124 and microRNA-9/9 to a cocktail of NEUROD2, ASCL1, and MYT1,35 generated mature human iNs capable of forming functional synapses with each other in the absence of mouse cortical neurons. The first method produced predominantly excitatory neurons, whereas the latter generated a heterogeneous mixture of excitatory and inhibitory neurons; the molecular mechanisms contributing to these different neuronal identities remain unclear. Both methods showed efficiencies of approximately 10%, with substantial variability between fibroblast lines. iN populations remain extremely heterogeneous, consisting of nearly 90% non-neuronal cells, and the temporal and spatial identity of iNs, relative to the human brain, is unresolved.

The ability to generate neuronal populations of a specific subtype would be ideal for cell-based studies. Using pools of mDA-specific transcription factors, 2 groups have directly reprogrammed human fibroblasts into neuronal populations consisting predominantly of mDA neurons. Using the factors ASCL1, NURR1, LMX1A, and MASH1, induced mDA (iDA) neurons were generated from mouse and human fibroblasts;36 these iDA released dopamine and were functionally integrated when transplanted into embryonic mouse brains. By enlarging the cocktail to ASCL1, NURR1, LMX1A, PITX3, FOXA2, and EN1, mouse iDAs were produced with gene expression profiles more closely resembling mDA neurons.37 Approximately 10% of the original fibroblasts were induced to iDAs; furthermore, upon transplantation of iDAs into the striatum of lesioned mice, striatum DA levels increased and function in a mouse model of PD improved. We anticipate that new protocols will soon be reported for the generation of additional neuronal subtypes.

Faster than hiPSC reprogramming and differentiation, iN production is now a robust method, but technical limitations remain when considering using this platform for cell-based studies of schizophrenia. First, iN generation bypasses neuronal development, eliminating the ability to test cellular phenotypes such as neural migration, specification, or maturation, which may be critical to disease progression. Second, this method results in terminally differentiated neurons, limiting the cellular material available for studies. To combine the strengths of hiPSC and iN systems, many groups sought a method to directly generate induced neural progenitor cells (iNPCs) from fibroblasts, in order to produce a substantial supply of neurons for study (table 3).

Direct Induction of iNPCs From Fibroblasts

The induction of iNPCs from fibroblasts provides, in a manner that better recapitulates neural development, a limitless supply of neurons for models of psychiatric disease. This year, 5 groups reported the generation of iNPCs from fibroblasts. The reports fell loosely into 2 different strategies: (1) incomplete hiPSC reprogramming in conjunction with growth in neural conditions and (2) overexpression of neural transcription factors.

Partial sets of reprogramming factors can generate iNPCs. iNPCs generated by inducing constitutive expression of SOX2, KLF4, and c-MYC in the presence of transient OCT4 differentiated into populations of up to 30% neurons or nearly 100% astrocytes, whereas iNPCs obtained by overexpression of OCT4, SOX2, and KLF4 in the presence of another pluripotency gene, ZIC3, differentiated into astrocytes, oligodendrocytes, and motor neurons but not central nervous system neurons. Notably, both these methods result in iNPCs that ultimately down-regulate pluripotency genes and upregulate NPC markers.

Several combinations of neural transcription factors appear to be sufficient to generate iNPCs, although at present all reports have required persistent long-term expression of induction factors to maintain iNPC identity. Three factors—SOX2, BRN2, and FOXG1—can induce direct conversion of mouse fibroblasts into tripotent iNPCs capable of generating neurons, astrocytes, and oligodendrocytes; however, expression of just SOX2 and FOXG1 produced mainly neuron-restricted progenitors, and expression of just FOXG1 and BRN2 generated progenitors of oligodendrocytes and immature neurons incapable of action potentials.40 A larger combination of factors—SOX2, BRN2, NR2E1, BM1, HES1, HES5, and c-MYC—could also convert mouse fibroblasts into iNPCs, although mature neurons from these iNPCs did not subject to electrophysiological validation.41 Most recently, a third group used just 1 factor, SOX2, to generate tripotent iNPCs that could differentiate to mature neurons capable of action potentials, from both mouse and human fibroblasts.42 It should be noted that SOX2-iNPC generation is markedly less efficient, requiring...
selection via three rounds of neurosphere suspensions. Generating iNPCs with just SOX2, however, opens the possibility of patterning iNPCs to specific identities by inducing via SOX2 in conjunction with other subtype-specific transcription factors.

iNPC technology provides a fast and robust protocol to obtain proliferative neural precursors and generates more homogeneous populations than current iN methods, all while bypassing time-consuming iPSC generation. Most iNPC protocols have been validated in vivo by functional integration of iNPC-derived neural cells into mouse brains.38-40 (None of the studies reported any cases of tumor formation by iNPCs.) At the moment, the major technical limitation to be addressed in iNPC generation is the adoption of integration-free induction techniques. New methods to permit patterning of specific regional identities are also critical. As the efficiency, purity, and patterning of iNPC methods advance, the adoption of these methods will provide a limitless source of neurons for the in vitro study of schizophrenia (table 4).

### hiPSC-, iNPC-, and iN-based Models of Schizophrenia

Although no reported studies have yet characterized iNPCs or iNs from patients with schizophrenia, a number of groups, including ours, have now published studies of schizophrenia-hiPSC neurons (figure 1). The first-generated schizophrenia-hiPSCs were from patients with a DISC1 mutation,45 but patient-derived neurons were not characterized. We reported neuronal phenotypes of hiPSC neurons from four patients with complex genetic forms...
of schizophrenia and showed that schizophrenia-hiPSC neurons had reduced neuronal connectivity and altered gene expression profiles.7 Although nearly 25% of the genes with altered expression had been previously implicated in schizophrenia, we also identified a number of new pathways that may contribute to schizophrenia. A third group observed a 2-fold increase in extra-mitochondrial oxygen consumption, as well as elevated levels of reactive oxygen species in NPCs derived from hiPSCs from one schizophrenia patient.44 Together, these studies offer an excellent proof of concept that reprogramming-based disease models can be used to study schizophrenia, although given the inherent variability between schizophrenia patients, more scalable methods to enable studies of larger numbers of patients need to be developed. Findings from all 3 studies await replication across larger patient cohorts.

At present, these cell-based studies only recapitulate molecular and cellular phenomena related to schizophrenia resulting from an underlying genetic defect; therefore, patient cohorts are typically selected on the basis of a known genetic defect or strong family history of schizophrenia. Technical constraints currently limit the patient cohort size for which neurons can be generated and characterized in vitro; cell-based studies remain necessarily small for the time being. Because of the clinical and genetic heterogeneity of this disorder, these methods may currently be most appropriate for the study of patients with a particular genetic or clinical commonality. For example, cell-based studies might be particularly useful in testing the effect of a rare copy number variant such as 22q11.2 or the genetic basis of clozapine responsiveness. The first strategy parallels traditional mouse-based studies of schizophrenia that investigate the effects of rare loci, whereas the second takes full advantage of the ability of these cell-based methods to investigate complex genetic disorders without full knowledge of all the

### Table 4. Summary of Methods of iNPC Generation

<table>
<thead>
<tr>
<th>Species</th>
<th>Transcription Factors</th>
<th>Patterning</th>
<th>NPC/Neuron Characterization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Sox2, Klf4, c-Myc, transient Oct4</td>
<td>Heterogeneous mixture of ventral fore, mid, and hindbrain fates</td>
<td>Immunostaining, gene expression profiling, qPCR, electrophysiology</td>
<td>Thier et al.38</td>
</tr>
<tr>
<td>Human</td>
<td>ZIC3, OCT4, NANOG, SOX2</td>
<td>Heterogeneous differentiation into motor neurons, astrocytes, and oligodendrocytes</td>
<td>Immunostaining, global gene expression profile, qPCR</td>
<td>Kumar et al.39</td>
</tr>
<tr>
<td>Mouse</td>
<td>Sox2, FoxG1, Brn2</td>
<td>Mainly forebrain</td>
<td>Immunostaining, qPCR, FACS, electrophysiology</td>
<td>Lujan et al.40</td>
</tr>
<tr>
<td>Mouse</td>
<td>Six2, Brn2, Nr2e1, Bmi1, Hes1, Hes5, c-Myc</td>
<td>Heterogeneous differentiation to neurons, astrocytes, and midbrain dopaminergic neurons</td>
<td>Immunostaining, qPCR</td>
<td>Tian et al.41</td>
</tr>
<tr>
<td>Mouse, human</td>
<td>SOX2</td>
<td>No specific neuronal fate</td>
<td>Immunostaining, qPCR, gene expression profiling, methylation patterns, electrophysiology</td>
<td>Ring et al.42</td>
</tr>
</tbody>
</table>

Note: NPC, neural progenitor cell; qPCR, quantitative PCR; FACS, fluorescence-activated cell sorting.
genes involved. Both are valid approaches; the first studies the effect of a single gene, the second studies the effect of genetic background.

When considering using induced cells to study schizophrenia, it is important to keep in mind the limitations of hiPSC-derived and induced neurons. Cell-based models presume that hiPSC-derived or fibroblast-induced neurons are “similar enough” to those in the human brain to be informative. Populations are typically comprised of neurons of mixed spatial identities and maturity and also may be incompletely patterned. Despite these important caveats, we believe that these new methods of modeling will result in insights into the initiation, progression, and treatment of schizophrenia.

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References


