Modeling Predisposition to Schizophrenia, a Genetically Heterogeneous Neuropsychiatric Disorder, Using Induced Pluripotent Stem Cells

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Introduction

Schizophrenia (SZ) is a debilitating yet relatively common psychiatric disorder, affecting approximately 1% of the world population. Genetic and epigenetic variations underlie differences in clinical outcome and treatment responsiveness (Campbell et al., 2008; Akbarian, 2010; McClay et al., 2011; Ferentinos and Dikeos, 2012; Ota et al., 2012; Hasan et al., 2013). However, the explanation for the 41–65% discordance rate of SZ between monozygotic twins sharing identical genetic predisposition to disease remains unclear (Cardno and Gottesman, 2000).

Environmental stressors, such as cannabis use, maternal immune activation, and birth complications, may also contribute to SZ (Brown, 2011; Connor et al., 2012; Scherr et al., 2012; Torrey et al., 2012), and recent efforts have attempted to combine animal models of the disease with environmental stressors (Kannan et al., 2013). Although these studies provide important insights into biological mechanisms that underlie at least part of the pathology of SZ, it is still difficult to fully recapitulate the heterogeneity of the disease and address the mechanisms of this "human" condition. Studies based on human induced pluripotent stem cells (hiPSCs) have the potential to combine both environmental and genetic influences by using cells from patients with known genetic backgrounds (Brennand et al., 2014a).

Generation of Neuronal Subtypes

Directed differentiation

Directed differentiation is an *in vitro* strategy that mimics *in vivo* development by applying small molecules and/or morphogens that mimic the signaling involved in the patterning, specification, and commitment of defined cell types during embryonic development. Treatment of human embryonic stem cells (hESCs) with Noggin (a bone morphogenetic protein [BMP] inhibitor) and SB431542 (a transforming growth factor beta [TGF-β] inhibitor), so-called "dual SMAD inhibition," directed 80% of hESCs into a population of neural stem and progenitor cells within 1 week, as assayed by PAX6 and *HES5*-eGFP (enhanced green fluorescent protein) reporter expression (Chambers et al., 2013). Dual SMAD inhibition is a remarkable method for rapidly differentiating neural populations from hiPSCs, thereby expediting the timeline and purity of neuronal differentiation protocols.

Dopaminergic neurons

Dopamine (DA) neurons in the midbrain (mDA neurons) are associated with distinctive neurological disorders, such as Parkinson's disease (PD), SZ, and attention deficit hyperactivity disorder (ADHD) (Sillitoe and Vogel, 2008). Dual SMAD inhibition followed by sonic hedgehog (SHH), BDNF, FGF8, and ascorbic acid treatment generates tyrosine hydroxylase (TH)–positive neurons from hiPSCs within 1 month (Chambers et al., 2009). This adherent culture protocol bypasses the embryoid body (EB) stage between hiPSC and neural rosette, and produces three times more TH^+ neurons (30%) than the conventional EB protocol (10%) (Boyer et al., 2012). Recent yields have been reported to be as high as 80% (Kriks et al., 2011). Overexpression of progerin (which facilitates cellular aging) in hiPSC-derived mDA neurons derived from PD patients and healthy controls reveals distinctive PD phenotypes, such as reduction of TH^+ neurons, dendrite degeneration, and enlarged mitochondria specifically in "geriatric" PD hiPSC mDA neurons (Miller et al., 2013).

Glutamatergic neurons

Glutamatergic neurons in the cerebral cortex, generally represented by pyramidal neurons, are projection neurons that relay information to remote areas of the cerebral cortex and other regions of the brain. Aberrant neuronal connectivity and function of the glutamatergic neurons are believed to increase susceptibility to neuropsychiatric disorders such as autism spectrum disorder (ASD) and SZ (de Bartolomeis et al., 2014). Synergistically with dual SMAD inhibition, activation of retinoic acid signaling (to restrict dorsal forebrain development) specifies hESCs and hiPSCs into cortical stem cell and progenitor cells expressing *FOXG1* and *EMX1*, which are markers for dorsalized forebrain (Shi et al., 2012). The cortical stem cell and progenitor cell population generates 70% class III β-tubulin–positive neurons by 40 d of differentiation; by 50 d, these neurons express *VGLUT1*, a glutamate synaptic vesicle marker with excitatory synaptic properties in electrophysiology (Shi et al., 2012). An alternative methodology, utilizing FGF2 and inhibitors of BMP, WNT/β-CATENIN, and TGF-β/ACTIVIN/NODAL pathways, also induces hiPSCs into neural progenitor cells with forebrain fate, which can be further differentiated into presynaptic ($SYNAPSIN1^+$) and postsynaptic ($PSD95^+$) excitatory cortical neurons (Mariani et al., 2012). When hESC-derived cortical progenitor cells and cortical pyramidal neurons were transplanted into the cortex of neonatal mice, engrafted cells sent axonal-like projections to multiple brain regions, where they established functional synapses and microcircuits with the host brain, suggesting the functional potential of

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cortical glutamatergic neurons directly differentiated from hiPSCs (Espuny-Camacho et al., 2013).

GABAergic neurons

In the cerebral cortex, GABAergic interneurons are implicated in neuropsychiatric diseases such as epilepsy, seizure, ASD, and SZ, likely owing to their essential role in fine-tuning and integrating the neural network (Marin, 2012). Cortical GABAergic interneurons initially arise from medial ganglionic eminences (MGEs) of the developing telecephalon, and only subsequently do they migrate into the neocortex (Anderson et al., 2001). To generate MGE neural progenitor cells with potential GABAergic identity, combinatorial inhibition of dual SMAD (SB431542 and LDN-193189) and WNT (XAV939) can be combined with late activation of SHH and differentiated hiPSCs into 80% *NKX2.1*-GFP–positive cells, a marker of ventral cells in the developing forebrain MGE (Maroof et al., 2013). If cocultured with mouse cortical neurons, these *NKX2.1*-GFP neurons show physiological activity consistent with GABAergic interneurons, and they further differentiate into somatostatin (SST), parvalbumin (PV), and calbindin-positive GABAergic interneurons. In parallel, a similar set of chemical cocktails (dual SMAD inhibition, WNT inhibition [DKK1], and SHH signaling activation) (Nicholas et al., 2013) also gave rise to neurons expressing GABAergic markers. These neurons had functional synaptic properties consistent with GABAergic neurons and were capable of functionally integrating into the microcircuitry of the host brain. Recent data suggest that the addition of caudalizing signal by FGF8 enhances the yield of *NKX2.1*-GFP^{+} cells more than WNT inhibition and SHH activation do alone (Kim et al., 2014).

Generation of hiPSC-derived neurons directed by morphogens and small molecules appears to faithfully reproduce the differentiation process into neuronal subtypes, similar to *in vivo* development, though a number of limitations of "directed differentiation" persist. First, the supply of recombinant growth factors may not be economically suitable for methodology such as massive high-throughput screening. Second, inefficient signaling activation by chemicals can restrain researchers from the precise combinational modulation required for proper differentiation. Third, the obstacle of spatially and temporally impure and heterogeneous neural subtype specification has not been overcome. Fourth, directed differentiation yields neurons that are immature relative to those in the human brain, with transcriptional profiles most resembling human fetal tissue (Mariani et al., 2012; Nicholas et al., 2013; Brennand et al., 2015). Finally, directed differentiation protocols require an extended time course of neuronal differentiation—up to 3 months—leading to slow experiment turnaround. Recently, "neuronal induction" has been shown to be a viable alternative strategy that addresses many of these concerns.

Neuronal induction

Patient-derived somatic cells can now be rapidly and directly converted from differentiated cells into neurons. During neurogenesis, a series of proneuronal transcription factors orchestrates the global gene expression network required for cell-fate specification, driving the cellular transition from neural stem/progenitor cells to mature neurons. Expression of key neurogenic regulators is sufficient to induce donor fibroblasts into neurons (iNeurons). In 2009, the Wernig group demonstrated that three proneuronal transcription factors—*Ascl1*, *Brn2,* and *Myt1l* (BAM)—directly converted mouse fibroblasts into heterogeneous but functional neurons in just 20 d (Vierbuchen et al., 2010). In humans, combining *NeuroD1* with these three BAM factors induced neurons from human fibroblasts, even though these iNeurons formed fully functional excitatory synapses only when cocultured with mouse primary cortical neurons (Pang et al., 2011). Expression of two micro RNAs (miR-9 and miR-124) with *ASCL1, MYT1L,* and *NEUROD2* improved neuronal induction efficiency, yielding iNeurons with electrical synaptic properties independent of the primary neuron coculture (Yoo et al., 2011).

Induced glutamatergic neurons

Expression of neurogenin 2 (Ngn2), a dorsal telencephalic fate determinant, transdifferentiates cortical astroglial cells into glutamatergic neurons with functional synapses *in vitro* (Berninger et al., 2007; Heinrich et al., 2010) and hiPSCs into functional iNeurons (Zhang et al., 2013). When combined with selection for *Ngn2* expression, more than 90% of cells express MAP2 (mitogen-activated protein 2), a dendritic marker, within 14 d, and, when cocultured with mouse cortical neurons, they elicit electrical characteristics of excitatory synaptic function within 21 d. Furthermore, Ngn2 iNeurons express the glutamatergic synaptic proteins VGLUT2, PSD95, and SYNAPSIN1 and successfully integrate when transplanted into a mouse brain.

Induced dopaminergic neurons

Combinatorial transduction of BAM factors, together with LMX1A and FOXA2 (fate determinants of mDA neurons), yields mDA-like cells that express

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TH but show poor functionality (Pfisterer et al., 2011). This may reflect the fact that BRN2 (one of the BAM factors) is enriched in pyramidal neurons (Dominguez et al., 2013). Accordingly, transduction of just *Ascl1*, *Lmx1b*, and *Nurr1* in mouse astrocytes yielded TH^+ neurons that secrete DA (Addis et al., 2011). Similar sets of transcription factors induced functional mDA neurons from both mouse and human fibroblasts, which expressed DA machinery components such as VMAT2, DAT, ALDH1A1, and CALBINDIN (Caiazzo et al., 2011; Kim et al., 2011a). Recently, transduction of *ASCL1*, *LMX1A*, and *NURR1* (ALN) yielded populations of 60% pure DA neurons (TH^+, class III β-tubulin$^+$ double neurons) from hiPSCs within 14 d (Theka et al., 2013).

Induced GABAergic neurons

Induction into GABAergic neuronal fate has only just begun. Expression of *Ascl1* together with *Dlx2*, a factor essential for GABAergic neuronal differentiation, is sufficient to transdifferentiate within 21 d mouse astroglial cells to synapse-forming neurons that are positive for GABAergic neuronal markers, including GAD67, calretinin, and vGAT (Heinrich et al., 2010). More recently, coexpression of miR-9 and miR-124, together with MYT1L and three transcription factors enriched in the developing striatum (*BCL11B*, also known as *CTIP2*, *DLX1*, and *DLX2*), induced human fibroblasts into a population analogous to striatal medium spiny neurons (Victor et al., 2014). These induced GABAergic neurons fire action potential trains with a long delay to initial spike, and if transplanted into the mouse brain, extend projections to the anatomical targets of medium spiny neurons.

One of the major concerns about "neuronal induction" is whether forced expression of neuronal transcription factors will overcome disease-specific deficits in neuronal patterning and/or maturation. An important proof of concept was the demonstration that iNeurons recapitulate the expected AMPA receptor–mediated excitation deficits when generated from mice with neuroligin-3 mutations, reminiscent of the neuronal phenotypes observed in primary neurons from these same mutant mice (Chanda et al., 2013). This finding strongly supports the utility of iNeurons for disease modeling. In contrast with directed differentiation, overexpression of transcription factor facilitates rapid conversion of somatic cells and iPSCs into a variety of functional neuronal subtypes in a dramatically shorter period of time. Furthermore, fate regulator–mediated induction orchestrates a more uniform conversion process in donor cells, giving rise to relatively homogenous populations of neuronal subtypes. However, the necessity of coculture with other neurons or glial cells to enable synaptic maturation remains an important challenge when considering applying iNeurons to high-throughput screening for drug development.

Using hiPSCs to Link Genetics and Cellular Phenotypes of Schizophrenia

Schizophrenia is ideal to study using hiPSC because it is a complex genetic disorder with no single causal mutation. Once neural progenitor cells or neurons are generated from patient hiPSCs, cellular phenotypes can be investigated to gain insight into the cellular mechanisms of SZ. Chiang et al. were the first group to derive and characterize hiPSCs from SZ patients alongside controls (Chiang et al., 2011). Follow-up studies by our group and others examined the neuronal phenotypes from neurons derived from patient hiPSCs. These neurons display decreased neurite outgrowth and synapse formation (Brennand et al., 2011; Robicsek et al., 2013; Wen et al., 2014) and impaired neurotransmitter release (Hook et al., 2014); in contrast, patient-derived neuronal progenitor cells exhibit defects in neuronal migration (Brennand et al., 2014b), deficits with adherens junctions (Yoon et al., 2014), and impaired mitochondrial function (Paulsen et al., 2011; Robicsek et al., 2013; Brennand et al., 2015).

Initial studies of hiPSC-derived neurons were limited by the availability of patient fibroblasts, and therefore, these findings represent a heterogeneous patient population in which little is known about the patients' genetic or clinical background. Follow-up studies are beginning to take two distinct approaches. The first is to characterize the cellular phenotype of clinically homogeneous patient cohorts. This approach enables researchers to learn about disease mechanisms that are shared across patients who exhibit similar symptoms or have similar drug response profiles. This type of study may lead to finding commonly disrupted pathways that could be relevant across a large subset of SZ patients.

The second approach is to examine a cohort of patients all carrying the same genetic mutation, such as a copy number variant (CNV) (Yoon et al., 2014) or point mutation (Wen et al., 2014). This approach is similar to mouse models because it links cellular phenotypes to pathogenic risk variants. This approach was used to uncover a cellular phenotype of the 15q11.2 CNV; hiPSCs developed from a patient with a 15q11.2 microdeletion displayed

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deficits in adherens junctions and apical polarity (Yoon et al., 2014). This approach was also used to study a CNV at 7q11.23, where deletion results in Williams–Beuren syndrome, whereas duplication causes 7q-microduplication syndrome. Examination of patient-derived hiPSCs and the differentiated cell types showed dosage-dependent alterations in disease-relevant transcriptional circuits (Adamo et al., 2015). This group also investigated the role of a transcription factor, GTF2I, which is disrupted by the CNV, and found that manipulating GTF2I caused a significant proportion of the transcription dysregulation created by the entire CNV (Adamo et al., 2015).

Another useful tool for this second approach is genome editing technology, such as CRISPR/Cas9 (CRISPR signifies clustered regularly interspaced short palindromic repeats; Cas9 is a class of RNA-guided endonucleases). This technology provides a simple, efficient approach for introducing genetic mutations, correcting genetic mutations (Cong et al., 2013; Jiang et al., 2013), and even enhancing (Maeder et al., 2013) or repressing (Larson et al., 2013) gene expression (Hsu et al., 2014). For example, in order to confirm that the synaptic defects observed in two psychiatric patients resulted from the identified *DISC1* frameshift mutation, Wen et al. produced isogenic hiPSC lines, both engineering the *DISC1* mutation into a control hiPSC line and repairing the mutation in a *DISC1* patient hiPSC line. In this way, they showed precisely that mutant *DISC1* causes synaptic vesicle release deficits and dysregulates the expression of many genes related to synapses and psychiatric disorders in hiPSC-derived forebrain neurons (Wen et al., 2014).

CRISPR/Cas9 enables investigation into the casual relationship between genotype and neuronal phenotypes because isogenic control lines can be derived, leaving the rest of the patient's genome unchanged, to ensure that a particular genetic variant is casual to the cellular phenotype (Martinez et al., 2015). Patients with the same variant but different genetic backgrounds can help researchers understand the contribution that additional risk alleles make to a cellular phenotype. Even the role of single genes within a CNV can be theoretically assessed in patient-derived hiPSCs by using CRISPR-Cas9 to replace or delete copies of single genes while maintaining the rest of the disrupted locus. Such studies could help determine the genetic elements and cell types that are most important for patients with SZ who carry a known neuropsychiatric risk allele, leading to more-targeted treatment of these patients.

Constraints of hiPSC-Based Studies

In designing hiPSC-based studies, it is important to be aware that genetic mutations and epigenetic misremodeling can occur during the reprogramming process. First, both CNVs (Laurent et al., 2011; Liu et al., 2014; Lu et al., 2014) and somatic coding mutations (Gore et al., 2011) will change the donor DNA. Importantly, more CNVs are present in early-passage hiPSCs than in higher-passage hiPSCs, implying that most novel CNVs generated during the reprogramming process are lost before any neuronal differentiation would occur (Hussein et al., 2011). Across 22 hiPSC lines reprogrammed using five different methods, each contained an average of five protein-coding point mutations, though at least half of these reprogramming-associated mutations previously existed in fibroblast progenitors at low frequencies (Gore et al., 2011).

Second, at the epigenetic level, evidence now demonstrates that aberrant DNA methylation remodeling (Lister et al., 2011; Nazor et al., 2012; Ma et al., 2014) and an erosion of X chromosome inactivation (Mekhoubad et al., 2012; Nazor et al., 2012) can occur in hiPSCs. Consistent with this finding, evidence suggests that donor cell type can influence the epigenome and differentiation potential of hiPSCs (Bar-Nur et al., 2011; Kim et al., 2011b). These genetic and epigenetic effects contribute to the "intraindividual variation" observed in hiPSC-based studies that exists because each hiPSC line generated from a given person will show subtle differences in gene expression and propensity toward neural differentiation.

Conversely, "interindividual variation" represents biological differences between individuals and can be addressed by studying ever-larger cohorts of patients and controls, thereby better capturing the heterogeneity among individuals. Unpublished data by our group and others suggest that intrapatient variability is less than interpatient variability. Therefore, well-designed and controlled experiments are critical to ensuring that researchers can draw meaningful conclusions from hiPSC-based studies of psychiatric disorders. To this end, we recommend that at least three hiPSC lines be compared per individual, in order to reduce the likelihood that a rare genetic or epigenetic mutation might affect disease-specific hiPSC lines in a meaningfully different way than they affect control hiPSC lines.

When investigating the effect of a single disease-associated allele, whether in the context of a simple Mendelian disorder or a complex genetic disease, an

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alternative to increasing cohort size is to compare isogenic hiPSC lines. In fact, a burst of recent hiPSC-based studies has used isogenic controls to demonstrate the precise effects of a single gene on neural phenotypes or gene expression (Liu et al., 2012; Wen et al., 2014).

Perhaps most critically, although recent studies have reported the importance of heritable genetic factors to neuropsychiatric disease and modeled the correlation between these risk factors and disease phenotype, it is still challenging to unravel the causality of environmental risk factors such as stressful life events, social anxiety, and neurotrauma (Howes et al., 2004). These factors remain infeasible to recapitulate in existing cell-based systems *in vitro*. Moreover, the question remains as to how to link relatively simple cellular phenotypes from hiPSC-derived neurons with the complex behavioral phenotypes of neuropsychiatric patients, which encompass delusions, hallucinations, negative affect, and impaired cognition. One strategy will be to build increasing complexity into hiPSC-based models. Future models will necessarily incorporate neuronal circuits that comprise at least two distinct neuronal cell types, synapsed in a defined orientation, together with oligodendrocytes to provide myelination, and astrocytes and microglia to incorporate critical aspects of inflammation and synaptic pruning. Circuits will need to be stimulated repeatedly to establish plasticity and exposed to meaningful levels of stress hormones and other environmental factors. A second strategy will be to transplant each of these relevant human cell types (neurons, astrocytes, oligodendrocytes, and microglia) into mouse models of disease, yielding increasingly humanized platforms for study. The ultimate solution, of course, will be in pursuing all the strategies we have discussed in tandem: larger cohorts, isogenic controls, improved patterning, and maturation of a variety of human neural cell types, cultured either as artificial circuits or transplanted into mice. Although models, by definition, must always lack the intricacies of human disease, the goal of hiPSC scientists should always be to strive for ever-increasing complexity in their models.

Limitations Due to Clinical Heterogeneity

The sample size of hiPSC-based studies remains relatively very small compared with the standards of genome-wide association studies of complex genetic disorders. Although ultimately, methods will have to be developed to permit comparisons among thousands of patients, to date, technical constraints have greatly limited hiPSC generation, neural progenitor cell differentiation, and cellular phenotyping (listed in order of increasing difficulty). Consequently, given the small sample size (typically one to four patients) of recent hiPSC-based studies, a major concern is whether the findings are representative of the larger patient population. Interpatient variability results from the heterogeneity among patients with SZ. As mentioned earlier, there are two strategies for hiPSC disease modeling of heterogeneous patient populations: (1) using a genetically homogenous patient cohort that share a single genetic lesion and characterizing the effect relative to isogenic lines generated through gene targeting technologies; and (2) selecting a patient cohort on the basis of a shared clinical phenotype and comparing them with individuals without the phenotype. The first strategy parallels traditional mouse-based studies of SZ that investigate the effects of rare loci, while the second takes full advantage of the ability of hiPSC-based studies to investigate complex genetic disorders without full knowledge of all the genes involved.

By modeling specific aspects of SZ rather than capturing the entire diversity of this disorder, researchers might be able to reduce the interpatient variability in hiPSC-based studies. In the short term, researchers can address this problem by selecting homogenous patient cohorts characterized by common genetic mutations, or by shared neurophysiological endophenotypes and/or pharmacological responses. Neurophysiological characterization of patients with SZ has identified abnormal responses to paired auditory stimuli (Geyer et al., 1990; Freedman et al., 1997) and defects in oculomotor control (Radant et al., 1997), though growing evidence suggests these endophenotypes may be heritable (Greenwood et al., 2012). Although strong evidence supports the pharmacogenetics of lithium-responsive bipolar disorder (McCarthy et al., 2010), new data support the heritability of antipsychotic resistance in SZ as well (Ota et al., 2012). Of course, additional factors ranging from epigenetic effects to circuit-based plasticity (derived from experience) may contribute to the heterogeneity of SZ. Nonetheless, though such risk factors are likely to be lost as part of the reprogramming process, we predict that some (even if not all) key mechanisms contributing to SZ can be studied using hiPSCs.

Multiple levels of etiology might contribute to SZ, increasing from biochemical to cellular to neuronal network and brain circuits. As the complexity of this "causal action" grows, it will become more difficult to resolve biological meaning through cell-based

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models (Kendler, 2013). Critics may also question the relevance of months-old *in vitro*–derived neurons in the study of SZ, a condition whose hallmark symptom of psychosis typically appears in late adolescence. For this reason, we posit that current hiPSC-based approaches may be most appropriately used in the study of SZ predisposition.

Future Considerations and Conclusions

Future advancements in developing new therapeutics for SZ, or any other neurological disorder, will rely on an understanding of commonly disrupted pathways. These pathways can be uncovered through several methods:

- Careful integration of human genetic studies to identify disease-related variants;
- Studying mouse models to examine the function of a variant in a complex *in vivo* environment; and
- Investigating patient-derived hiPSC models to determine the casual role of a variant on a cellular phenotype and the contribution made by complex genetic interactions.

Incorporating this type of information from studies investigating CNVs in SZ has begun to implicate specific pathways, such as genes regulating NMDA receptors and ARC (activator recruited cofactor) complexes (Purcell et al., 2014; Szatkiewicz et al., 2014). This knowledge can inform the development of new therapeutics. However, the potential of hiPSCs does not end with mechanistic studies but rather continues into clinical trials where hiPSCs can be used as a complementary human *in vitro* component.

Before the full potential of hiPSCs can be realized, a large amount of work remains to be done. Optimized protocols for more-homogeneous populations of hiPSCs and pure populations of differentiated cells need to be fully developed and validated. Doing so will decrease the time and cost needed to develop patient-derived hiPSC models, allowing for more patient lines to be examined. Improved culturing techniques should be applied to form more complex neural circuitries that better resemble the human brain. This is currently an area of great interest in the context of the development of human cortical spheroids (Paşca et al., 2015). Finally, experiments investigating complex diseases should be designed to collect multilayer data, including the patient's clinical information, genome, transcriptome, proteome, and epigenome (Schadt et al., 2014). Gathering this type of multilayered information is now possible with the advancements being made in sequencing technology and bioinformatics. From this information, we can begin to build networks and identify the key drivers of complex genetic disorders such as SZ.

Acknowledgments

Parts of this paper are excerpted from other publications. The sections "Generation of Neuronal Subtypes" and "Constraints of hiPSC-Based Studies" are excerpted from Ho et al., 2015, From "directed differentiation" to "neuronal induction": modeling neuropsychiatric disease. Biomark Insights 10(Suppl 1):31–41. The section "Limitations Due to Clinical Heterogeneity" is excerpted from Brennand et al., 2014a, Modeling heterogeneous patients with a clinical diagnosis of schizophrenia with induced pluripotent stem cells. Biol Psychiatry 75:936–944. Finally, the sections "Using hiPSCs to Link Genetics and Cellular Phenotypes of Schizophrenia" and "Future Considerations and Conclusions" are excerpted from Flaherty and Brennand, 2015 (manuscript in preparation).

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