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Transcriptomics Analysis of iPSC-derived Neurons and Modeling of Neuropsychiatric Disorders

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Abstract

Induced pluripotent stem cell (iPSC)-derived neurons and neural progenitors are great resources for studying neural development and differentiation and their disruptions in disease conditions, and hold the promise of future cell therapy. In general, iPSC lines can be established either specifically from patients with neuropsychiatric disorders or from healthy subjects. The iPSCs can then be induced to differentiate into neural lineages and the iPSC-derived neurons are valuable for various types of cell-based assays that seek to understand disease mechanisms and identify and test novel therapies. In addition, it is an ideal system for gene expression profiling (i.e., transcriptomic analysis), an efficient and cost-effective way to explore the genetic programs regulating neurodevelopment. Moreover, transcriptomic comparison, which can be performed between patient-derived samples and controls, or in control lines in which the expression of specific genes has been disrupted, can uncover convergent gene targets and pathways that are downstream of the hundreds of candidate genes that have been associated with neuropsychiatric disorders. The results, especially after integration with spatiotemporal transcriptomic profiles of normal human brain development, have indeed helped to uncover gene networks, molecular pathways, and cellular signaling that likely play critical roles in disease development and progression. On the other hand, many challenges remain in the usage of iPSC-derived neurons for modeling neuropsychiatric disorders, for example, how to generate relatively homogenous

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populations of specific neuronal subtypes that are affected in a particular disorder and how to better address the genetic heterogeneity that exists in the patient population.

Keywords

Neuropsychiatric Disorders; iPSCs; Transcriptome; RNA-seq

Neuropsychiatric disorders are complex brain disorders caused by multiple genetic and environmental factors. Genome-wide association studies (GWAS), copy number variation (CNV) analyses, and exome sequencing have successfully identified a few hundreds of genetic risk factors (Cross-Disorder Group of the Psychiatric Genomics, 2013; Fromer et al., 2014; Gulsuner and McClellan, 2015; Gulsuner et al., 2013; Major Depressive Disorder Working Group of the Psychiatric et al., 2013; Purcell et al., 2014; Ripke et al., 2013; Ruderfer et al., 2013; Schizophrenia Psychiatric Genome-Wide Association Study, 2011; Schizophrenia Working Group of the Psychiatric Genomics, 2014; St Clair, 2009; Sutrala et al., 2007; Sutrala et al., 2008; Szatkiewicz et al., 2014). Despite these exciting advances, the pathophysiology of neuropsychiatric disorders has long been challenging to study in the laboratory. One major problem is the lack of suitable preclinical models for studying underlying genetic mechanisms, recapitulating molecular and neurodevelopmental pathways, and drug screening. In the past decades, many animal models (e.g. murine and primate models) have been developed for investigating specific risk genes and modeling particular behavioral or pathological phenotypes (Nestler and Hyman, 2010). While they have provided valuable and significant insights into the roles of specific genes and signaling pathways, they have inherent limitations for accurately recapitulating the complexity of human brain development and function, thus hindering the translation of critical research findings into novel therapeutics (Dragunow, 2008). Direct assays of human brains or specific regions are feasible (e.g., fMRI), but manipulation and functional interruption are obviously impractical. It is also considered unethical, or at least difficult, to develop *in vitro* neuronal cell lines from biopsies of patient brains. Postmortem human brains offer opportunities for studying the pathophysiology and molecular defects, but postmortem studies have their own technical challenges and confounding factors, such as premorbid medical conditions, cause of death, postmortem delay in sample processing, cellular heterogeneity, and poor RNA quality (Chailangkarn et al., 2012; Kretzschmar, 2009; McCullumsmith and Meador-Woodruff, 2011).

Generation of iPSCs for studying neuron development and functions

One way to circumvent these limitations is to use pluripotent stem cells (PSCs), which include embryonic stem cell (ESCs) and induced pluripotent stem cell (iPSCs), the latter of which can be derived from patients, to study neural differentiation *in vitro* and model the dysregulated processes occurring in patients. Human ESCs (hESCs) were first established in 1998 (Thomson et al., 1998). The discovery has revolutionized regenerative medicine and cell biology research. Human iPSCs (hiPSCs) were established a decade later (Takahashi et al., 2007; Takahashi and Yamanaka, 2006) by reprogramming adult somatic cells with four transcription factors (OCT4, SOX2, cMYC and KLF4). Since then, various combinations of

transcription factors, miRNAs, and small molecules have been developed to reprogram different types of somatic cells to iPSCs, with increased efficiency, accelerated kinetics, and reduced safety concerns (Malik and Rao, 2013). The recently developed small molecule approach is particularly appealing as it eliminates many of the drawbacks of the previous methods, such as introduction of exogenous pluripotency-associated elements (Bar-Nur et al., 2014). Other developments include the use of integration-free reprogramming with episomal vectors (Okita et al., 2011), mini-circle DNA vectors (Jia et al., 2010), direct protein delivery (Kim et al., 2009) and piggyBac transposons (Woltjen et al., 2009). For studying complex processes and pathological states including neural development and neuropsychiatric disorders, these non-integrating technologies for generating iPSCs are preferred because the effects of random retroviral integration on differentiation can be avoided (Prilutsky et al., 2014).

PSCs can be differentiated into desired cell types and thus offer invaluable and practical resources for studying the development and function of many cell types (Peitz et al., 2013), including neurons (the focus of current review), providing powerful tools for *in vitro* disease modeling. Direct lineage reprogramming (i.e., “transdifferentiation”), by which one adult somatic cell type is transformed into another lineage without undergoing an intermediate pluripotent state (Graf and Enver, 2009), has advantages over iPSC-based approach, but it has its own challenges, like low reprogramming efficiency and reduced competence for synapse formation of the induced neurons (Pang et al., 2011; Pfisterer et al., 2011; Son et al., 2011) (Figure 1). Compared to ESCs, iPSCs have two major advantages in disease modeling. First, the fact that ESCs can only be established from embryos limits their application to study complex genetic diseases. More importantly, although some genetic variants have a strong effect on phenotypes (strong penetrance), most neuropsychiatric disorders likely arise from multiple genetic insults (Cross-Disorder Group of the Psychiatric Genomics et al., 2013; Gaugler et al., 2014; Hersen et al., 2011), which are retained in patient-specific hiPSCs, allowing investigators to study the effect of specific genetic factor(s) and its interaction with patient-specific genetic background in terms of penetrance and severity under different environmental conditions. In this regard, patient-specific hiPSCs have advantages over genetically engineering approaches (e.g., deletion of a gene or a chromosomal region), because even if the engineered change is introduced into multiple ESC lines, they may not necessarily harbor the genetic background in patients (Siller et al., 2013). From these perspectives, hiPSCs serve as an important bridge linking studies in model organisms to investigations of human postmortem brains, by providing live cells, including iPSCs and their differentiated neural progenitor cells (NPCs) and neurons, with the complex genetic backgrounds that exist in patients. In fact, the number of studies using patient-specific iPSC-derived neurons to model neuropsychiatric disorders has been increasing rapidly (Table 1 and 2). These studies have provided critical new insights into the pathological of neuropsychiatric and neurodevelopmental disorders.

Transcriptomics characterization of hiPSC-derived NPCs and neurons

Although there are many molecular and cellular assays for studying iPSC-derived neural cultures and identifying the functional differences between patient and control samples, we will focus on gene expression analysis in this review, as cell identity and function can be

characterized at the molecular level by unique transcriptomic signatures. Furthermore, to better understand high phenotypic variability in iPSC systems and to assess their appropriateness for disease modeling, more and more researchers have started to rely on quantitative measures rather than qualitative ones (Bock et al., 2011; Lin et al., 2011). Gene expression is one of the better quantitative metrics. In fact, the assessment of the specific neuronal identity of PSC-derived cells mainly relies on a number of NPC or neuronal markers, as it is conventionally accepted that the expression (or absence) of these markers is correlated with specific types of neurons, their maturation stages and functions. The most frequently used molecular markers for neuron identity are *TUBB3* and *MAP2* (Table 1). However, accurate classification of samples into specific neuronal subtypes and specific maturation stages needs to assess the expression of a number of genes, including transcription factors and neurotransmitter receptors. For instance, the expression of specific neurotransmitter receptors, synaptic proteins and ion channels (e.g. *GAD67* for GABAergic neurons; *VGLUT1* for glutamatergic neurons; *TH* for dopaminergic neurons) is frequently examined in the analysis of heterogeneous population of neuronal cultures (Pacca et al., 2011).

While individual neural markers, especially in combination, provide critical gene signatures for neural lineage and functions, transcriptome-wide analysis, carried out by either microarray or RNA-seq, is more comprehensive and can lead to additional insightful findings. As microarray or RNA-seq analysis allows researchers to generate a quantitative index of transcriptomes, with tens of thousands of genes being profiled simultaneously, a transcriptome profile can serve as a more robust and stable quantification than other phenotypic measurements, and its characterization may help to identify multiple sources of variations in iPSC generation (Marioni et al., 2008; Schmid et al., 2012) and to better understand the merits and drawbacks of individual iPSC systems.

The first study using RNA sequencing analysis to obtain transcriptomic profile of iPSC-derived neurons showed that early differentiating neurons derived from hiPSCs underwent an extraordinary array of quantitative changes in gene expression (Lin et al., 2011), similar to the findings in hESC systems (Wu et al., 2010), including the dramatic increase in expression of many neuronal genes, such as *STMN4*, *GRIA2* and *CARTPT*. Importantly, the RNA-seq transcriptomic data can be used to evaluate the progression of *in vitro*-differentiation of the iPSC-derived neurons (Stein et al., 2014) and map the differentiating neurons to the temporal and spatial brain development regions based on the similarity of the transcriptomic profiles (Hjelm et al., 2013). Overall, these studies demonstrated the power of transcriptomic analyses, especially in understanding the cell composition and differentiation stage of iPSC-derived neuronal cultures. Such an analysis can certainly lead to the discovery of new and more specific neural markers too.

Genome-wide analysis, however, also reveal some critical issues in the study of iPSCs and their differentiated cells. More specifically, hiPSCs display substantial differences from hESCs with respect to the expression of hundreds of genes (Chin et al., 2009), genome-wide DNA methylation profiles (Doi et al., 2009), and neural differentiation potency (Hu et al., 2010), with some of the differences reflecting the origin of the somatic cells used for reprogramming, due to incompleteness in erasing the epigenetic features in the source cells.

This has raised the concerns about the equivalence of ESCs and iPSCs (Figure 1). Likewise, variation in efficiency of producing a desired neuron type between iPSCs and ESCs has raised the question of whether iPSCs are capable of generating functional neurons as efficiently as ESCs (Hu et al., 2010) (Figure 1). Comprehensive transcriptomic profiling has helped us address these concerns (Bock et al., 2011). Despite that iPSC outliers showed significantly different expression profiles from the ESCs and they often had low efficacy of neural differentiation, iPSCs and ESCs have high degree of global similarities in gene expression. In light of this, a bioinformatics tool (“scorecard”) was developed to help researchers predict the feasibility of specific iPSC lines with respect to their capacity to differentiate into neurons (Bock et al., 2011).

Transcriptomics modeling of neuropsychiatric disorders with hiPSCs

Only rarely will genetic alteration at a single gene fully account for a neuropsychiatric disorder (monogenic model). More commonly, a small number of genes will each have a small independent effect on increasing risk, but interact together to cause a disorder (polygenic model). In addition to providing data to better understand the high phenotypic variation in iPSC systems and their appropriateness for the study of brain disorders, transcriptomic analysis allows a global and unbiased identification of potential molecular alterations critical to neuronal differentiation and development. In this regard, RNA-seq analysis provides many advantages over microarray, including the detection of low-copy transcripts, novel transcripts, long non-coding RNAs (lncRNAs), and splice isoforms (Feng et al., 2011; Mortazavi et al., 2008). This is especially important, considering the critical roles played by cell type-specific splicing in neuronal differentiation and the growing list of lncRNAs that have a role in neurogenesis (Barry et al., 2014; Hattori et al., 2008; Mercer et al., 2008; Mercer et al., 2010; Qureshi and Mehler, 2012). Note that it is still very valuable to study rare single-gene variants (or disease candidate genes), as they are excellent starting points to understand the biological processes in more complex forms of the disease. In fact, gene expression analysis of key neural genes, as opposite to global transcription profiling, in iPSC-derived samples from psychiatric patients has provided important insights into potential deficits in neural development in patients, and their roles in the pathogenesis of neuropsychiatric disorders. The resulted findings can often be corroborated in subsequent transcriptomic analyses in which the expression or function of key candidate genes is disrupted by genetic engineering approaches, as discussed below.

Autism Spectrum Disorders (ASDs)

ASDs are a collective terminology for a group of complex neurodevelopmental disorders characterized by impaired social interaction and communication, as well as the presence of restricted repetitive behaviors (Geschwind and Levitt, 2007; Zwaigenbaum et al., 2013). Functional disruptions of a few hundred genes have been implicated in ASDs (State and Levitt, 2011), including the methyl CpG binding protein-2 (*MECP2*) and *TRPC6* (a voltage-independent, Ca^{2+} -permeable cation channel involved in dendritic spine and excitatory synapse formation) (Tai et al., 2008; Zhou et al., 2008). By establishing iPSC-derived neurons from an ASD individual with a *de novo* balanced translocation t(3;11)(p21;q22) that disrupts the *TRPC6* gene, investigators recently demonstrated that reduction in *TRPC6*

expression resulted in disruption of neuronal development, morphology and function (Griesi-Oliveira et al., 2015). In addition, the investigators found that *TRPC6* was regulated by *MECP2*, and thus suggested a connection between two ASD candidate genes. The work demonstrates the value of using iPSC-derived neurons for studying and modeling diseases caused by rare sporadic variants. Likewise, two recent studies of ASD-specific hiPSCs, their differentiated neurons, and telencephalic organoids (DeRosa et al., 2012; Mariani et al., 2015) showed that dysregulation of neuronal differentiation occurs in ASD, based on abnormal expression of many differentiation-associated genes, including *DCX*, a marker of neuronal precursor cells and immature neurons, *OTX2*, an anterior neuronal marker whose expression is normally induced by retinoic acid, and *FOXG1*, a known transcription factor important for telencephalon development. The latter study further confirmed that atypically high expression of *FOXG1* was responsible for the phenotypic abnormalities by restoring some normal neurobiological activities in ASD-patient specific iPSC-derived neurons with *FOXG1* knockdown.

As a complementary approach for studying the genetic disruptions in ASD, researchers have knocked down (KD) or knocked out (KO) ASD candidate genes in neurons derived from hiPSCs that were originally generated from healthy individuals (i.e., control hiPSC lines) and studied the functional consequences under isogenic condition (Figure 2). Novel findings emerging from the analysis of the transcriptional targets of ASD candidate genes are a good testimony to the value of transcriptome analysis. For instance, chromodomain helicase DNA-binding protein 8 (*CHD8*) is one of the strongest genetic risk factors for ASD. Its known molecular function as a DNA helicase does not immediately reveal how it may be related to ASD. However, the discoveries of abnormal gene expression related to neural development, synaptogenesis, and other ASD-implicated pathways by *CHD8* KD or KO in proliferative neuronal stem cell or early differentiating neuronal progenitor cells, including iPSC-derived NPCs and neurons, help uncover the link (Cotney et al., 2015; Sugathan et al., 2014; Wang et al., 2015; Wilkinson et al., 2015). It was intriguing that although there were only limited overlaps in lists of differentially expressed genes across different studies, at the function and pathway levels genes involved in similar functions (e.g. cell communication and neuronal development) were affected by reduced *CHD8* expression in different contexts (Wang et al., 2015). In addition, these analyses found *CHD8*-regulated genes were significantly overlapped with the targets of other autism-risk genes, including *TCF4* (Chen et al., 2014a) and *NRXN1* (Zeng et al., 2013). These results provided transcriptomic evidence that ASD candidate genes have overlapping function and converge on downstream regulatory pathways. Therefore, the value of such studies goes beyond the identification of targets and pathways that could be affected by individual ASD genes, by providing a more broad picture that ASD candidate genes are primarily involved in altered synaptic structure and disrupted neuron differentiation.

Rett Syndrome (RTT)

RTT is a monogenic syndrome due primarily to loss of function mutations in the *MECP2* gene, which causes intellectual disability and epilepsy, and often presents with autistic-like symptoms (Na et al., 2013). Considering the severity of symptoms and pathologies (Weng et al., 2011), an important first step in disease modeling RTT using patient-specific hiPSCs was

to demonstrate their capacity to differentiate into all three germ layers, which was accomplished by Hotta et al. (Hotta et al., 2009). The first study using RTT-specific iPSC-derived neurons confirmed the previously proposed regulatory role of *MECP2* on transcription and retrotransposition of Long Interspersed Nuclear Elements-1 (LINE-1 or L1s) (Muotri et al., 2010). LINE-1s are abundant retrotransposons that can impact human genome stability and genome-wide gene expression by a variety of mechanisms (Kazazian, 1998; Perepelitsa-Belancio and Deininger, 2003), especially in neurons (Coufal et al., 2009). A number of studies further characterized the phenotypic abnormality in RTT-specific iPSC-derived neurons in terms of morphology, synapse connectivity and glutamatergic synaptogenesis (Ananiev et al., 2011; Cheung et al., 2011; Kim et al., 2011; Marchetto et al., 2010a). To test if the observed reduction in the number of glutamatergic synapses in RTT-specific hiPSC-derived neurons was directly related to *MECP2* mutation, Marchetto et al. compared wild-type-iPSCs derived neurons with *MECP2* knockdown, *MECP2* overexpression and controls, and confirmed that *MECP2* was crucial to glutamatergic synaptogenesis (Marchetto et al., 2010a). In addition, many differentially expressed genes between RTT iPSC neurons and controls were found.

Schizophrenia (SCZ)

SCZ is a neurodevelopmental disorder with a ~1% prevalence in the general population (Sullivan et al., 2003). Although the onset of schizophrenia generally manifests late in adolescence, it is widely believed that neuronal impairment often predates clinical symptoms by a prodromal period that can appear in childhood. There is strong evidence implicating dopaminergic, glutamatergic and GABAergic neurons in SCZ (Javitt et al., 2008), but the affected cell types and underlying molecular mechanisms remain elusive. The first study on SCZ-specific neurons reported diminished neuronal connectivity and altered expression of cAMP and WNT signaling pathways (Brennand et al., 2011), which is in line with current models of SCZ etiology. In addition, this transcriptomic analysis not only confirmed the major findings of pharmacological and GWAS studies, but also identified novel pathways not linked to SCZ previously, such as NOTCH signaling, cell adhesion and axon guidance. However, a new study found abnormal expression of genes related to cytoskeletal remodeling and oxidative stress in SCZ neurons (Brennand et al., 2015). These results probably reflect the complexity of SCZ, with multiple impaired neural function pathways, as well as genetic heterogeneity in the SCZ hiPSC lines. Considering that molecular changes would be easier to uncover using neurons with a common and high penetrant genetic risk factor, we have been developing patient-specific hiPSCs and differentiating neurons from SCZ patients with 22q11.2 deletion, which can lead to velocardiofacial syndrome (VCFS) (Pedrosa et al., 2011). 22q11.2 deletion is the strongest known genetic risk factor for SCZ. Gene expression in early differentiating neurons uncovered dysregulation of a number of transcription factors, chromatin remodeling factors, and synaptic proteins relevant to SCZ pathogenesis, including *ZNF804A* and *NRXN1* (Pedrosa et al., 2011), as well as disruption in MAPK signaling and apoptosis (manuscript under review). Importantly, our findings were consistent with abnormalities in these pathways found in SCZ (Lang et al., 2014; Pietersen et al., 2014; Sellmann et al., 2014; Winchester et al., 2012). In a follow-up experiment by knocking down *ZNF804A*, which is among the top candidates implicated in SCZ, ASDs and bipolar disorder (O'Donovan et al., 2008; Riley et al., 2010), in a control hiPSC lines and

examining the affected pathway by expression profiling, we found that down-regulated differentially expressed genes were enriched in interferon-signaling pathway, suggesting that *ZNF804A* might regulate the response of differentiating neurons to inflammatory cytokines (Chen et al., 2015). In an independent study, *ZNF804A* was also found to modify the expression of genes involved in cell adhesion (Hill et al., 2012).

The iPSC system can also be valuable for modeling environmental effects known to play a role in SCZ and ASD (Atladóttir et al., 2012; Li et al., 2009), such as maternal immune activation (MIA). Recently, we exposed iPSC-derived, 3-dimensional neuronal aggregates to heat shock (39°C for 24 hours), to mimic the fever that can accompany MIA, and carried out RNA-seq analysis (Lin et al., 2014). To our surprise, we observed a dramatic alteration on the expression of some SCZ and ASD genes, including *ARNT2* and *ZNF804A*. This suggests that some environmental factors like MIA might have a more protracted effect on gene expression and neurogenesis in the presence of underlying risk alleles that adversely affect the expression of genes in the same direction as the environmental insult. Interestingly, the iPSC-derived NPCs from SCZ patients were found to show increased cell-to-cell variation in the levels of HSF1 activation (as determined by HSP70 expression) by environmental challenges compared to controls (Hashimoto-Torii et al., 2014). These studies demonstrate the value of transcriptomic analysis and suggest a genetic connection among SCZ patients with 22q11.2 deletion, *ZNF804A* mutations, MIA, and dysregulated cytokine responses. As more transcriptomic analyses become available, the essential molecular pathways disrupted in SCZ and their connections will emerge.

Other neuropsychiatric disorders

In addition to these three common neuropsychiatric disorders, hiPSC derived neurons has been utilized to model a variety of others, including Fragile X syndrome (Bar-Nur et al., 2012; Liu et al., 2012b; Sheridan et al., 2011b), Timothy syndrome (Krey et al., 2013; Pa ca et al., 2011), Prader-Willi & Angelman (Chamberlain et al., 2010; Yang et al., 2010), and Bipolar Disorders (Chen et al., 2014b) (Table 1).

Beyond quantification of gene expression and differential expression analysis

RNA-seq data can also be analyzed for allele-biased expression (ABE), non-coding RNAs expression (including microRNAs), and post-transcriptional modification events like RNA-editing (Figure 2). Recent studies suggest that besides genetic factors both stochastic processes and imprinting can lead to monoallelic or allele-biased gene expression (ABE), the latter of which may be more widespread than previously considered (Gimelbrant et al., 2007). Thus, it is important to study ABE in brains and neurons, because ABE could help explain monozygotic (MZ) twin discordance and reduced penetrance in SCZ and perhaps ASD. Researchers recently indeed found a large number of genes expressed in an allele-biased manner in neural stem cell and differentiating neurons, and those genes were enriched for ASD and SCZ candidates (Jeffries et al., 2012; Lin et al., 2012). These findings indicate that allele-biased expression could be a common regulatory process that affects a variety of molecular and cellular pathway relevant to neurodevelopment.

Many studies have shown that microRNAs play significant roles in neurogenesis and synaptogenesis (Hsu et al., 2012; Jimenez-Mateos et al., 2012; Krichevsky et al., 2003; Liu et al., 2012a). Additionally, multiple lines of evidences from GWAS and molecular studies supported microRNAs' role in the pathogenesis of SCZ and other neuropsychiatric disorders, with miR-137 as the most notable example (Green et al., 2013; Schizophrenia Psychiatric Genome-Wide Association Study, 2011). We have recently carried out miRNA-seq analysis of differentiating neurons from iPSCs derived from controls and SCZ patients with 22q11.2 deletion that contains several miRNAs, and *DGCR8*, which codes for a critical component in the miRNA biogenesis pathway (Zhao et al., 2015). We found that the expression of miRNAs within the 22q11.2 deleted region were reduced to about one-half of the level observed in control neurons, and the expression of several additional neuronal miRNAs that do not map to 22q11.2 were also affected. The predicted targets of these miRNAs were enriched for neurological diseases and psychological disorders, as well as neuron development, axonogenesis, and other key neural pathways (Zhao et al., 2015).

Lastly, transcriptomic analysis can identify post-transcriptional modifications. RNA editing is one type of post-transcriptional event. The conversion of adenosine to inosine (A-to-I) is the major RNA editing process, and it is most active in human brains. Most RNA editing sites have been detected in RNA transcripts involved in neurotransmission (Sanjana et al., 2012). A number of brain specific microRNAs also undergo RNA editing (Nishikura, 2010). Interestingly, a comparative analysis of RNA editing on postmortem cerebella between autistic and healthy subjects found that RNA editing was much more extensive in the autism samples than controls (Eran et al., 2013), highlighting the importance of extending this type of analysis to other neuropsychiatric disorders. Whole transcriptomic analysis is also capable of detecting fusion genes (Zhang et al., 2014) and valuable for constructing co-expression networks (Gulsuner et al., 2013), which can help investigators uncover the gene networks and pathways disrupted in neuropsychiatric disorders.

Intergenic GWAS SNPs and ncRNAs

Unbiased transcriptome analysis has proven useful to uncover the biological significance of DNA variants (e.g., SNPs) identified in GWAS of neuropsychiatric disorders, since many of the variants are map to intergenic regions or large introns where regulatory ncRNAs may exist. In the characterization of gene expression change during the transition from iPSC to iPSC-derived neurons (Lin et al., 2011), we found that many lncRNAs exhibited substantial changes in expression. One of which is *HOTAIRMI*, a regulator of several *HOXA* genes during myelopoiesis (Zhang et al., 2009). These findings, combined with GWAS evidence, clearly suggested a role for these novel regulators in a subgroup of patients. An ncRNA (MSNP1AS) located near the site of an ASD associated SNP in chromosome 5p14.1 is another example (Kerin et al., 2012). While some ncRNAs may interact directly with protein-coding genes, for example, competing for miRNA targeting, many ncRNAs may have a role in regulating transcription at the chromatin level (Barry et al., 2014; Kocerha et al., 2015; Qureshi and Mehler, 2012), such as those RNAs transcribed from enhancers (eRNAs) (Kim et al., 2010). The connection of eRNAs to intergenic GWAS signals is currently being actively investigated, with more promising results to come.

Integration with spatiotemporal transcriptomic profiles of normal human brain development

It is important to connect findings from iPSC study to *in vivo* brain development. Several studies have related to neural gene expression profiles to the spatial and temporal transcriptome data in the BrainSpan Atlas (Kang et al., 2011), which provided gene or exon-level expression data from 16 regions of the human brains, sampled from 57 postmortem human brains and spanning from embryonic development to late adulthood stages. A recent analysis of gene expression in iPSC-derived NPCs from SCZ patients found that both control and SCZ NPCs shared similar spatiotemporal expression profiles to 6-week-old neurons in the BrainSpan data (Brennand et al., 2015). Moreover, the differentially expressed genes between SCZ NPCs and controls exhibited enrichments for the co-expressed gene modules from hippocampus, medial prefrontal cortex, posterior superior temporal cortex, inferolateral temporal cortex, and striatum networks, which are brain areas suggested to be impaired in SCZ. It is worthy mentioning that other research groups have constructed spatiotemporal coexpression networks of a number of genetic variants associated with ASD or SCZ in the BrainSpan data and identified specific brain development periods and regions that were particularly vulnerable to gene disruption in the development of ASD or SCZ brains (Kang et al., 2011; Willsey et al., 2013). Both studies found significant contribution of coexpression networks in fetal development, supporting the neurodevelopmental hypothesis of ASD and SCZ.

The challenges: heterogeneity and variation

Although iPSC technology shows extensive promise, there are certainly issues that prevent it from being widely adopted to model complex disorders (Dolmetsch and Geschwind, 2011). Among them, one major limitation is the lack of reproducibility between clones from the same individual and between different individuals (Bock et al., 2011; Hu et al., 2010; Prilutsky et al., 2014; Telias and Ben-Yosef, 2014). We should note that this limitation is actually not so unique to iPSCs but more broadly applied to *in vitro* systems based on cell cultures. Heterogeneity could come from imperfect reprogramming during the development of iPSCs, and variation in the efficiency of neuronal differentiation. For instance, recent work showed variable neural differentiation efficiency of hiPSCs (Hu et al., 2010) and various states of functional maturation of iPSC-derived neurons, using the same pipeline for iPSC generation (Marchetto et al., 2010a). These differences undoubtedly alter the properties of the iPSC-derived neurons. Therefore, generation and selection of iPSCs lines that can efficiently and reliably differentiate into the desired neuron types is critical in the modeling of neuropsychiatric diseases.

As mentioned earlier, a variety of cell types have been used for iPSC reprogramming, but skin fibroblasts have been the predominant source for neuropsychiatric disorders so far (Table 1). Peripheral blood mononuclear cells are a good alternative. From the biological perspective, dental pulp is an appealing source for making iPSCs for modeling neuropsychiatric disorders because of its developmental origins from ectoderm, specifically neural crest cells (Miletich and Sharpe, 2003). With this in mind, we have compared the

transcriptomes of iPSCs and their differentiated neurons that were generated either from skin fibroblasts (F-iPSC) or dental pulp (T-iPSC) (Chen et al., 2013). Interestingly, despite a high degree of correlation in gene expression profiling between the neurons derived from the two different sources, we observed a reduction in expression of genes involved in hindbrain development (e.g., *IRX1* and several *HOX* genes) and an increase in expression of some key forebrain transcription factors in (e.g., *FOXP2* and *OTX1*) in T-iPSCs derived neurons. This finding suggests that iPSC-derived neurons originated from different somatic tissues/cells could behave differently for modeling neuropsychiatric disorders (Figure 1).

Fundamentally, neuronal differentiation is a complex process, typically resulting in a mixed neuron cell population, even using well-controlled experimental protocols. Such lack of uniformity in neuronal identity has led to concerns as to whether iPSC-derived neurons will be able to produce a pathological phenotype reliably and stably, and subsequently how drug screening or toxicity trials based on iPSC-derived disease models will correlate with clinical response (Hanna et al., 2010; Stadtfeld and Hochedlinger, 2010) (Figure 1). Importantly, the common practice of assessing neuronal identity based on a small number of genes may underestimate the heterogeneity in a neuronal culture population. Indeed, studies utilizing genome-wide expression analysis have shown that within-clone iPSCs lines may exhibit as great variation as between-clone lines (Chen et al., 2013; Chen et al., 2015; Lee et al., 2009), an observation not detected by expression levels of few classic markers (e.g., *TUBB3*, *MAP2*, *PAX6*, and *SYN1/2*), because they displayed no differences in expression. The immediate question following the concern about the identities of iPSC-derived neurons is what features of *in vivo* development can be appropriately recapitulated in the process of *in vitro* neural differentiation (Figure 1).

In addition to the variation related to neuronal differentiation, there is genetic heterogeneity in the iPSC lines. While this may be advantageous in that it captures the true genetic complexity of the patient population, it also introduces challenges in comparing results from different iPSC lines. For example, *CHD8* was recently knocked down in NPCs or NPC-like cells (SK-N-SH) in three independent studies (Cotney et al., 2015; Sugathan et al., 2014; Wilkinson et al., 2015). The lists of differentially expressed genes from those studies exhibited marginal overlaps, although they are all enriched with neural functions. The limited overlap can be partially due to the genetic difference among the cell lines that were used. Using the same neural culture conditions and increasing sample sizes are two of the necessary steps needed to address these issues.

Perspective: reduce variation and beyond neural culture

Researchers have started to deal with the issue of heterogeneity in neuronal cultures derived from pluripotent stem cells by developing strategies to produce more homogeneous neuronal cultures. This is critical not only for disease modeling, but also for therapeutic purposes. Reliable production of homogeneous dopaminergic neurons has been suggested as a top priority for mechanistic studies and cell-based therapies in Parkinson disease; many studies of this disease indeed have benefitted from improved neuronal purification (Doi et al., 2014; Woodard et al., 2014). More efficient protocols to differentiate iPSC cells into relatively homogeneous populations of neurons are emerging. The recently developed protocol for

differentiating excitatory glutamatergic cortical neurons by overexpressing neurogenin-2 (Ngn2) is highly promising for modeling diseases, such as SCZ (Zhang et al., 2013). This protocol successfully converts human ESCs and iPSCs into functional neuronal cells with almost 100% yield and purity. An alternative option to reduce heterogeneity in experiments is cell sorting using distinct surface markers. On the other hand, SCZ and other neuropsychiatric disorders cannot be explained on the basis of any single neuroanatomical structure or neurotransmitter system, and ultimately multiple neuronal cell types need to be analyzed. Single cell RNA-seq is also a promising option, as described in a recent study that evaluated the heterogeneity of the adult human cortex, which resulted in the identification of new neuron subtypes (Darmanis et al., 2015). Transcriptomic data from thousands of individual cells can serve as a reference cellular map to assist the identification of specific markers for neuronal, glial, and vascular cells with distinct connectivity and electrophysiological properties at different locations in the brain. Very encouragingly, this work showed that the expression patterns of a few known genes are sufficient for defining subtypes of neuronal cells. We foresee an increase in the utilization of single cell RNA-seq to identify neuronal cell types from its success in unbiased cell-type classification (Pollen et al., 2014).

It should be noted that although homogenous populations of neuronal cultures offer many technical advantages, they nevertheless do not reflect the actual conditions in developing brains. In fact, more complex 3-dimension models, despite containing a mixture of different cell types, could better recapitulate many aspects of brain development, like the formation of the cerebral cortex and discrete cortical layers, and thus provide investigators with novel insight into the pathophysiology of neuropsychiatric diseases, in which deficits may not be manifested in a monotypic cell type (Figure 1). Mariani et al. has demonstrated that hiPSC could be differentiated into multilayered structures that showed a similar gene expression profile as the embryonic telencephalon (Mariani et al., 2012). More recently, iPSCs were used to generate 3-dimension (3-D) cerebral organoids that also resembled the first trimester telencephalon (Lancaster et al., 2013). These studies open a new avenue to model brain development using hiPSCs and to study developmental dysregulation using patient-specific hiPSCs, especially for the identification of abnormal gene expression in specific brain regions or structures that are dramatically different between humans and mice. In addition, these models of the human telencephalon can be used to study potential gene $\times$ environment interactions that adversely affect the developing brain (Lin et al., 2014). We foresee in the near future that investigators will be able to apply single cell RNA-seq to the 3-D organoids to simultaneously monitor cell population change and detect gene expression difference (within individual cell types) between disease samples and controls.

Conclusions

The iPSC technology provides a very promising tool for creating *in vitro* models of neurodevelopmental disorders. Gene expression analysis using patient-specific iPSC-derived neurons or control iPSC lines with perturbed expression of candidate genes provides an excellent opportunity to go from individual genetic variations to molecular pathways and cellular networks for tackling the complexity of psychiatric disorders.

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Figure 1. Overview of the pipeline to generate iPSC-derived neurons and related issues in the application of iPSCs for modeling neuropsychiatric disorders

A) equivalence of iPSCs and ESCs; B) identities and mixture of iPSC-derived neurons; C) difference between *in vivo* development and *in vitro* differentiation; D) effect of source tissues on iPSCs-derived neurons; E) neuronal aggregates vs neuronal culture; F) directed reprogramming from somatic cells to neurons vs iPSC as intermediate step.

Figure 2.
Outline of transcriptomics comparison using iPSC system and downstream bioinformatics analyses.

Table 1

List of Reports on Modeling Neuropsychiatric Disease using iPSC-derived Neurons

Disease	Reference	Source of Cells	Stage of Neuronal Differentiation (Identity)	Neuronal Markers	Transcriptomics Profiling
Autism	Griesi-Oliveira et al. (2015)	Dental pulp	Neural progenitor cells and cortical neurons	<i>TUBB3, MAP2, SYNI, CTIP2, TBR1</i>	Microarrays
Autism	DeRosa et al. (2012)	PBMC	Neurons (GABAergic)	<i>TUBB3, MAP2, NCAM, VGAT, GAD67, Synapsin</i>	No
Autism	Mariani et al. (2015)	Fibroblasts	Neurons (Telencephalic Organoid)	<i>NESTIN, PAX6, BRN2, SOX1/2, DCX and TUBB3</i>	RNA-seq
Bipolar disorder	Chen et al. (2014b)	Fibroblasts	Neurons	<i>MAP2, TUBB3</i>	Microarrays
Fragile X syndrome	Sheridan et al. (2011a)	Fibroblasts	Neural progenitor cells	<i>NES, SOX1</i>	No
Fragile X syndrome	Bar-Nur et al. (2012)	Fibroblasts	Neurons	<i>NCAM</i>	No
Fragile X-associated tremor/ataxia syndrome	Liu et al. (2012b)	Fibroblasts	Neurons	<i>TUBB3, MAP2, Synapsin, VGLUT1, GPHN</i>	No
Lesch-Nyhan syndrome	Mekhoubad et al. (2012)	Fibroblasts	Neurons	<i>TUBB3, MAP2</i>	No
Phelan-McDermid syndrome	Shecheglovitov et al. (2013)	Fibroblasts	Neurons	<i>MAP2</i>	No
Prader-Willi & Angelman	Chamberlain et al. (2010)	Fibroblasts	Neurons	<i>TUBB3, MAP2</i>	No
Prader-Willi & Angelman	Yang et al. (2010)	Fibroblasts	Neurons	<i>TUBB3, MAP2</i>	No
Rett syndrome	Muotri et al. (2010)	Fibroblasts	Neural progenitor cells	<i>TUBB3</i>	No
Rett syndrome	Marchetto et al. (2010a)	Fibroblasts	Neurons	<i>TUBB3, MAP2, Synapsin, VGLUT1, GABA</i>	Microarrays
Rett syndrome	Cheung et al. (2011)	Fibroblasts	Neurons	<i>TUBB3, MAP2</i>	No
Rett syndrome	Ananiev et al. (2011)	Fibroblasts	Neurons	<i>TUBB3</i>	No
Rett syndrome	Kim et al. (2011)	Fibroblasts	Neurons	<i>TUBB3</i>	No
Rett syndrome	Amenduni et al. (2011)	Fibroblasts	Neurons (Glutamatergic, GABAergic)	<i>VGLUT1, VGLUT2, GAD67, MPA2, TBR1</i>	No
Rett syndrome	Ricciardi et al. (2012)	Fibroblasts	Neurons (Glutamatergic)	<i>TUBB3, MAP2, VGLUT1, TH, GAD65, PSD95, VGAT</i>	No
Schizophrenia	Brennand et al. (2011)	Fibroblasts	Neurons (Glutamatergic, GABAergic, Dopaminergic)	<i>VGLUT1, GAD67, TH, TUBB3, MAP2</i>	Microarrays
Schizophrenia	Pedrosa et al. (2011)	Fibroblasts	Neurons (Glutamatergic)	<i>VGLUT1, TUBB3</i>	Microarrays
Schizophrenia	Brennand et al. (2015)	Fibroblasts	Neural progenitor cells, Neurons	Forebrain transcription factors	Microarrays

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Disease	Reference	Source of Cells	Stage of Neuronal Differentiation (Identity)	Neuronal Markers	Transcriptomics Profiling
Timothy syndrome	Pa ca et al. (2011)	Fibroblasts	Cortical neurons (glutamatergic, GABAergic)	N/A	Microarrays
Timothy syndrome	Krey et al. (2013)	Fibroblasts	Cortical neurons (glutamatergic, GABAergic)	MAP2	No

Table 2
List of Reports on Modeling Neuropsychiatric Disease using iPSC-derived Neurons with Conditional Treatment and Transcriptomics Profiling.

Target disease	Treatment	Reference	Source of Cells	Stage of Neuronal Differentiation (Identity)	Neuronal Markers	Transcriptomics Profiling
Autism	Knockout of <i>CHD8</i>	Wang et al. (2015)	Fibroblasts	Neural progenitor cells/Neurons	<i>TUBB3</i>	RNA-seq
Autism	Knockdown of <i>CHD8</i>	Sugathan et al. (2014)	Fibroblasts	Neural progenitor cells	<i>PAX6, SOX, MSII</i>	RNA-seq
Autism and schizophrenia	Knockdown of <i>NRXN1</i>	Zeng et al. (2013)	Fibroblasts	Neural stem cells	N/A	RNA-seq
Schizophrenia	Knockdown of <i>ZNF804A</i>	Chen et al. (2015)	Fibroblasts	Neurons (Glutamatergic)	<i>TUBB3</i>	RNA-seq
Schizophrenia	Heat-shock at 39°C for 24 hours	Lin et al. (2014)	Fibroblasts	Telencephalon	Forebrain transcription factors, <i>SLC17A6, SLC17A7</i>	RNA-seq
Rett syndrome	Knockdown/overexpression of <i>MeCP2</i>	Marchetto et al. (2010b)	Fibroblasts	Neurons	<i>TUBB3, MAP2, Synapsin, VGLUT1, GABA</i>	Microarrays