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MINI-SYMPOSIUM: Using iPSCs to understand human neurological disease: potential and limitations

# Understanding neurodevelopmental disorders using human pluripotent stem cell-derived neurons

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Keywords - disease modeling, human pluripotent stem cells, neurodevelopment, neuronal differentiation, psychiatric disorders.

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**Abstract**

Research into psychiatric disorders has long been hindered by the lack of appropriate models. Induced pluripotent stem cells (iPSCs) offer an unlimited source of patient-specific cells, which in principle can be differentiated into all disease-relevant somatic cell types to create in vitro models of the disorder of interest. Here, neuronal differentiation protocols available for this purpose and the current progress on iPSCs-based models of schizophrenia, autism spectrum disorders and bipolar disorder were reviewed. We also discuss the impact of the recently developed CRISPR/Cas9 genome editing tool in the disease modeling field. Genetically engineered mutation of disease risk alleles in well characterized reference “control” hPSCs or correction of disease risk variants in patient iPSCs has been used as a powerful means to establish causality of the identified cellular pathology. Together, iPSC reprogramming and CRISPR/CAS9 genome editing technology have already significantly contributed to our understanding of the developmental origin of some major psychiatric disorders. The challenge ahead is the identification of shared mechanisms in their etiology, which will ultimately be relevant to the development of new treatments.

## INTRODUCTION

Neurodevelopmental disorders are a group of conditions typically manifest early in life and are characterized by developmental deficits that produce impairments of personal, social, academic or occupational functioning. Neurodevelopmental (or neuropsychiatric) conditions include autism spectrum disorders, intellectual disabilities, schizophrenia and bipolar disorder, and have a substantial genetic component (7, 19, 66, 104).

For a long time, psychiatric research mainly relied on post-mortem studies and animal models. The first approach has the major limitation of representing the end-stage of the disease, without being informative about its origin and pathological progression. More-over, changes observed in post mortem tissues may be secondary effects of a patient’s prolonged use of medications. Animal models, predominantly rodents, are available for several psychiatric disorders, especially autism spectrum disorders and schizophrenia, but they have so far failed to show significant predictive validity for drug discovery (56, 71). This may be because of the inability of model organisms to represent unique higher human functions and consequently, to recapitulate all the symptoms characterizing a particular disorder (63). For example, despite the numerous rodent models available

for schizophrenia, these are mostly representative of the psychotic aspects of the disease, but do not reliably reproduce the cognitive and negative symptoms, such as impaired working memory, anhedonia and social withdrawal (37). In the case of bipo-lar disorder, there are no animal models available to represent both the manic and depressive extremes characterizing the disorder (11).

In just a decade from the derivation of the first line (84, 85), induced pluripotent stem cells (iPSCs) have become a fundamental tool for modeling human development and diseases, as well as for drug discovery. Like their mouse counterparts, human iPSCs are believed to have the same self-renewal and pluripotency properties of human embryonic stem cells (hESCs), but are derived (reprogrammed) from somatic cells, such as skin fibroblasts (84), keratinocytes (1), dental pulp (86, 98) or blood (48). The reprogramming is achieved by forcing the expression of key pluripotency genes such as OCT4, SOX2, c-MYC andKLF4insomatic cells, where the reprogramming factors start a self-regulatory loop that initiates and maintains pluripotency (58, 67). Expression of these reprogramming factors can be induced via viral transduction (6, 69), transfection of polycistronic plasmids (61), mRNAs (92) or direct delivery of recombinant proteins (103). More-over, treatment with specific combinations of small molecules has been shown to greatly increase the reprogramming efficiency (32, 78).

The use of adult somatic cells as starting material means that iPSCs are free from the ethical concerns that surround the use of hESCs, and that they can be derived from individuals carrying genetic variants that predispose to an increased risk of human dis-eases. Working on iPSCs, and PSCs in general, has become increasingly important in psychiatric research thanks to the ability to study the consequences of a large number of disease-associated mutations in the phenotypically relevant cell types. This is playing an important role in advancing our understanding of the cellular mechanisms underlying psychiatric disorders and reinforcing the hypothesis of their developmental origin.

## GENERATION OF DISEASE RELEVANT NEURONAL CELL TYPES FROM HUMAN PSCS

The brain contains thousands of neuronal types that differ in terms of neurotransmitter identity, electrophysiological properties and afferent/efferent connectivity (62). Different neurological diseases often exhibit pathologies specific to certain brain regions and/or cell types. This means that the possibility of observing and, there-fore, being able to correct a specific phenotype, is strictly dependent on the presence of the appropriate neuronal type in the system used. Therefore, an important consideration in modeling human neurological diseases is the generation of neural cell types targeted by the disease of interest.

A number of neuronal cell types have been implicated in psychiatric disorders: cortical projection neurons (23), inhibitory inter-neurons (23, 44), hippocampal neurons (25), dopaminergic neurons (23) and striatal medium spiny neurons (26, 80). Protocols to generate these neuronal types have been developed and are reviewed in this paragraph. Many lines of evidence demonstrate that in vitro PSC differentiation, to a large extent, mimics vertebrate development. In the context of neuronal conversion, PSCs firstly exit the pluripotent state and acquire a neuroectoderm fate. Therefore, the majority of neuronal differentiation protocols start with the induction of a specific regional neuroepithelial phenotype (region-specific progenitors) from which the target cells arise. This is mostly achieved by artificially recapitulating the signaling environment that the region-specific progenitors normally experience in vivo, by adding an appropriate combination of "inductive" molecules. The aim is to induce a cascade of transcription resembling normal

development, leading to the expression of a combinatorial set of transcription factors characteristic to the desired neural progenitor phenotype.

Currently, a popular method for generating neural cells from hPSCs is via monolayer differentiation protocol by dual SMAD inhibition (14). Normal central nervous system development follows an anterior first–posterior later temporal fashion. As such, the first neuroepithelial cells generated from PSCs exhibit features of forebrain regional identity. These anterior progenitors readily mature into neurons with predominantly cortical glutamatergic identity (9, 24, 79). Cell types of all cortical layers appear in a sequence reminiscent of in vivo corticogenesis, from deep layers to superficial ones. The efficiency of this approach was shown to be very high, with glutamatergic neurons accounting for 80% to nearly 100% of the cells in culture.

The specification of the other neuronal fates requires the use of additional morphogens to mimic the in vivo environment of the corresponding brain region. For example, the derivation of GABAergic interneurons requires the activation of sonic hedgehog (SHH) signaling (46, 47), in some cases combined with WNT inhibition (57, 64), to induce the medial ganglionic eminence (MGE) fate demonstrated by the expression of transcription factor NKX2.1. However, despite the induction of a high percentage of NKX2.11 MGE-like progenitors, efficient generation of mature interneurons, including the two major subtypes, somatostatin (SST) and parvalbumin (PV), have so far proved challenging. On the other hand, caudal ganglionic eminence (CGE)-derived interneurons expressing calretinin have been derived with a higher efficiency (>70%), by exposing late neural progenitors to activin A, a member of the TGF-b superfamily (13).

When applied to forebrain neural progenitors at an earlier time window, activin A induces the specification of a lateral ganglionic eminence (LGE) fate, leading to the production of 40%–50% of medium spiny neurons (4). Other strategies for deriving this type of neurons rely on the use of SHH, alone or in combination with WNT inhibition (45, 49, 65), in a similar way to MGE protocols. Dopaminergic neurons, which are born in the ventral midbrain, can be differentiated from hPSCs by exposing the neural precursors to different combinations of WNT agonists with SHH (20, 39, 41) or FGF8 (96). In addition, FGF signaling blockade using an ERK/MEK inhibitor on exit of pluripotency, followed by addition of SHH and FGF8 has also been reported to induce authentic mid-brain dopamine neurons (34). The yield of tyrosine hydroxylase positive cells is elevated in all these cases, reaching above 80%. Finally, only one protocol has been reported so far for the production of hippocampal neurons (101). For these cells, the application of WNT3a and BDNF to dorsal forebrain progenitors seems to be essential to induce the expression of PROX1, a marker for dentate gyrus hippocampal neurons.

Monolayer differentiation has proved itself a highly efficient and reliable paradigm for generating a number of neuronal types. Indeed, monolayer based forebrain glutamatergic neuron differentiation has been the platform of choice for the majority of disease modeling articles published so far (10, 11, 15, 30, 38, 53, 74, 77, 81, 94, 100). However, the monolayer culture system has limitations in visualizing potential alterations in the cytoarchitecture of the derived "brain tissue." This shortcoming can be overcome, to a certain extent, by differentiating PSCs in three-dimensional (3D) structures called organoids (43, 55, 72), although at the expense of higher variability, both within and between organoids, and in different preparations. To date, only Mariani et al have used telencephalic organoids for analyzing the developmental abnormalities with iPSCs derived from patients with idiopathic autism (54).

The generation of "induced neurons" (iNs) by direct reprogramming represents an alternative to conventional PSC neural differentiation. iNs are produced by forcing the expression of a defined set

of transcription factors crucial for the acquisition of neuronal fate in somatic cells or PSCs (2, 31, 42, 68, 99). iN protocols for the generation of specific neuronal types have also been published (12, 17, 70, 83, 89, 97). However, the iN approach may not be suitable for modeling diseases where pathogenesis occurs at neural progenitor stage, because it bypasses the process of neural progenitor specification, proliferation and differentiation choice toward distinct neuronal and glial fates. Moreover, in contrast to hPSC neural differentiation, the number of iNs that can be generated from the donor somatic cells is limited because of the restricted proliferative capacity of somatic cells prior to senescence. These shortcomings combined may explain why there are no studies published to date using iN technology to model psychiatric disorders.

## CURRENT STATUS OF IPSCS-BASEDRESEARCH FOR PSYCHIATRICDISORDERS

Brain imaging studies have demonstrated changes in the anatomy and neuronal activity in patients suffering from psychiatric disorders, while post-mortem studies have revealed aberrant cell-lar morphology (8, 22, 33, 95). The iPSC technology has provided an invaluable tool to investigate the cellular basis of such alterations and elucidate the molecular pathways that may be targeted for drug discovery.

The first proof-of-principle study using iPSCs was published by Brennand et al in 2011 (11). The authors reported that neurons derived from schizophrenic patients' iPSCs differ from those of the controls in neuronal connectivity, morphology and gene expression. This work was followed by several other schizophrenic iPSC studies reporting the emergence of earlier developmental abnormalities.

Robicsek et al differentiated schizophrenia and control iPSCs into glutamatergic and dopaminergic neurons and found defects in the maturation of both cell types, with the dopamine lineage more severely affected (74). The authors also reported differences in mitochondrial distribution and function. This aspect of the phenotype was more pronounced in dopaminergic progenitors than the glutamatergic cells. This could suggest the presence of a higher vulnerability for dopamine cells to oxidative stress, but it could also reflect, at least in part, their extremely impaired maturation.

Together with alterations of mitochondrial membrane potential and neuronal morphology, other defects often reported in neuronal cells from schizophrenic patients iPSCs are relative to WNT signaling and migration (10, 88) (see also Table 1).

All the studies listed above were based on heterogeneous cohorts of schizophrenic patients, selected only on the basis of their diagnosis, without knowledge of their genetic risk variants. This probably played a significant part in the high experimental variability evident in some of the results.

It is known that schizophrenia has a strong genetic component, with rare Copy Number Variations (CNVs) significantly increasing the risk of developing this disorder [reviewed by Kirov (40)]. Stratifying patients based on the presence of specific genetic mutations could help to reduce the degree of variability associated with iPSC work and discover new mechanisms that otherwise may be masked by the heterogeneity of the patients' samples.

Wen and colleagues took the genetics orientated approach by analyzing the effect of Disrupted In SChizophrenia 1 (DISC1) mutations, which are known to co-segregate with major psychiatric illnesses (60, 94). iPSC lines were derived from two patients carrying the same frameshift mutation in the DISC1 gene and three unaffected individuals. The authors reported altered morphology and electrophysiological properties in DISC1 neurons, as well as the expression of genes related to

synaptic transmission, neural development and major mental disorders. They also established the causality between DISC1 mutations and the changes observed by repeating some of the analysis in several isogenic cell lines. These were derived by correcting the DISC1 mutation in one of the mutant lines and by introducing the same frameshift deletion present in patients in two control lines. Both synaptic and vesicular release properties were restored to normal levels in the cell line in which the DISC1 gene sequence had been corrected, while the control lines carrying DISC1 deletion recapitulated the original mutant phenotype. However, the majority of CNVs associated to psychiatric disorders contain multiple genes. This genetic feature makes iPSC modeling particularly advantageous over the generation of animal models. Microdeletion of the 15q11.2 locus has been reported by several studies as an important risk factor for schizophrenia (82, 87). Yoon and colleagues demonstrated that iPSCs derived from 15q11.2 deletion carriers had significant defects in neural rosette formation (100). The 15q11.2 region contains four genes, CYFIP1 was proposed to be the likely responsible gene for the observed phenotypes. Indeed, increasing CYFIP1 expression by lentiviral transduction in differentiating 15q11.2del iPSCs rescued the abnormal expression of apical polarity markers, while reduction of CYFIP1 expression by shRNA in a control line mimicked the phenotype observed in the deleted progenitors. The authors extended their quest into CYFIP1 function by shRNA knockdown of Cyfip1 in mouse embryos via in utero electroporation, which resulted in incorrect localization of radial glia cells and nascent neurons. However, the consequences of CYFIP1 disruption in post-mitotic neurons were not explored.

As for autism spectrum disorders, in vitro modeling was first applied to syndromic autism, such as Rett syndrome (RTT) and Phelan-McDermid syndrome (PMDS). RTT syndrome is one of the most common causes of mental retardation mainly affecting girls and is caused by mutations of the methyl CpG binding protein 2 (MECP2) (3). iPSCs based investigations into RTT reported defects in neuronal maturation in patients' cells (38, 53) (see also Table 1). In particular, Marchetto et al demonstrated the presence of morphological alterations and reduced number of glutamatergic synapses in RTT neurons (53). This defect could be rescued by IGF1, a neurotrophic factor capable of promoting synaptogenesis. The same group subsequently reported the involvement of MeCP2 in regulating the expression of TRPC6, one of the genes disrupted by a translocation recently found in an autistic patient (30).

Similar cellular phenotypes were reported in in vitro models of PMDS. Shcheglovitov et al investigated the cellular phenotypes of iPSCs-derived neurons from two patients carrying heterozygous deletion of chromosome 22q13.3, the mutation responsible for PMDS (77). Cortical neurons derived from these iPSCs displayed impaired excitatory synaptic transmission, while the properties of their inhibitory synapses were not affected. These deficits could be either rescued by increasing the expression of SHANK3, a gene included in the deleted locus, or by IGF1 treatment, which did not affect SHANK3 levels.

Taking a different approach, Mariani et al derived iPSCs from four probands with idiopathic autism, carrying no known genetic mutation previously associated with autism spectrum disorders, and unaffected family members (54). Transcriptomic analysis of telencephalic organoids derived from patients and control cells revealed many differentially expressed genes, mainly relative to cell fate, proliferation, axonal guidance, synaptic function and ion channels. Consistently with these results, probands' cells showed dysregu-ated cell cycle and overproduction of inhibitory neurons, a phenotype that could be rescued by attenuating FOXG1 levels.

In the context of bipolar disorder, after performing a microarray analysis of iPSC-derived neurons from three patients and three controls, Chen and colleagues found a similar alteration in the expression of transcription factors regulating dorsoventral telencepahlic patterning (15). These

results, however, do not significantly overlap with those from Mertens et al, the only other publication employing iPSCs for bipolar disorder modeling to date (59). Their work, based on hippocampal neurons derived from bipolar patients, showed that the hyperexcitability and abnormal mitochondria size of these cells could be rescued by lithium treatment, while alteration of mitochondrial membrane potential could not be improved.

**Table 1.** Continued

| Disease | Genotype | Cellular model | Findings | Reference |
| --- | --- | --- | --- | --- |
| Syndromic autism (Phelan-McDermid syndrome) | 22q11.3 deletion | • iPSCs from 2 patients and 3 control PSCs lines (2 iPSCs and HUES9) <br>• Differentiation to cortical neurons | • Altered excitatory post-synaptic transmission (reduced amplitude and frequency of excitatory post-synaptic currents, EPSCs) <br>• Inhibitory synaptic transmission unaffected <br>• Synaptic deficits restored to control levels by lentiviral transduction increasing SHANK3 expression, or IGF1 treatment | Shcheglovitov et al (77) |
| Syndromic autism | 15q11-13 duplication <br>15q11-13 deletion (Angelman syndrome, AS) | • iPSCs from 4 duplication carriers, 1 AS patient and 1 control <br>• Embryonic bodies (EBs)-based differentiation to a mixed population of excitatory and inhibitory neurons | • Altered level of transcripts related to neuronal differentiation, cell cycle and protein catabolism <br>• Several autism and epilepsy candidate genes amongst the differentially expressed genes | Germain et al (28) |
| Autism spectrum disorder | Translocation in 3p21 and 11q22 chromosomes (Disruption of VPRBP and TRPC6 genes respectively) | • iPSCs from 1 patient and 2 control lines (1 iPSCs and 1 HUES6) <br>• Differentiation to forebrain NPCs and neurons | • Differentially expressed genes relative to nervous system development and function (several CREB-target genes) <br>• Alteration of $Ca^{2+}$ influx, morphology and synapses <br>• Normal phenotype restored by lentiviral-mediated WT TRPC6 expression or hyperforin (TRPC6 agonist) treatment <br>• Mutant phenotype mimicked by TRPC6 downregulation by shRNA <br>• Involvement of MeCP2 in TRPC6 regulation | Griesi-Oliveira et al (30) |
| Autism spectrum disorder | Mixed | • iPSCs from 4 patients and 8 unaffected first-degree family members <br>• Differentiation to cerebral organoids | • Altered expression of genes involved in cell fate, proliferation, axonal guidance, synaptic function and ion channels <br>• Faster proliferation of ASD iPSCs and NPCs <br>• Overproduction of GABAergic neurons <br>• Phenotype rescued by reduction of FOXG1 expression via shRNA | Mariani et al (64) |
| Bipolar disorder | Mixed | • iPSCs from 3 patients and 3 controls <br>• Differentiation to forebrain neurons | • Dysregulated expression of several transcripts regulating dorso-ventral telencephalic patterning, calcium signaling and miRNAs processing | Chen et al (15) |
| Bipolar disorder | Mixed | • iPSCs from 6 patients and 4 controls <br>• Differentiation to hippocampal dentate gyrus neurons | • Upregulation of mitochondrial genes, higher mitochondrial membrane potential and smaller mitochondria size in BD neurons <br>• Hyperexcitability (higher Na+ activation, increased action potential firing and amplitude) <br>• Mitochondrial and electrophysiological changes rescued by lithium (LI) treatment in a subset of neurons (from Li-responsive patients) | Mertens et al (59) |

In the interest of space, Table 1 summarizes the above works together with additional studies not individually discussed in this mini review. It is evident that differences exist between studies within the context of the same disorder. These discrepancies may attribute to different differentiation protocols, culture systems and analysis methods. Nevertheless, some common themes emerge from these studies. In general, genes involved in nervous system development are reported to be affected by many studies and, in line with this, several phenotypes are already present at the neural progenitor stage. In particular, altered WNT signaling and mitochondria dysfunction seem to be frequently reported in patient-derived cells (10, 11, 59, 74, 81, 88, 90). They may represent shared mechanisms in the etiology of neurodevelopmental psychiatric disorders.

**CRISPR-BASED GENOME EDITING AS A POWERFUL ALTERNATIVE TO MODELNEURODEVELOPMENTAL DISORDERS**

iPSCs represent a virtually unlimited and bankable source of patient-specific cells that can be differentiated into many disease-relevant neuronal types. Therefore, they allow the investigation of cellular phenotypes in cohorts of patients sharing a specific genetic mutation or the same clinical manifestation with unknown genetic background. This is particularly advantageous in the context of those disorders for which a genetic cause has not been identified, such as idiopathic autism cases (5). However, iPSC-based studies can suffer from high variability because of the differences in the genetic background of different patients, reprogramming methods and culture conditions (75). Working with genetically modified hESCs or a well characterized reference line of hiPSCs and their isogenic controls can represent an alternative to the use of patient iPSCs. This strategy will avoid the variability linked to the different genetic backgrounds of distinct patient iPSC lines and reduce the necessary work load of studying multiple patient iPSC lines required for identifying true phenotype and establish causality.

The CRISPR (Clusters of Regularly Interspaced Short Palindromic Repeats)/Cas9 technology allows genome editing more easily and efficiently than traditional gene targeting via Homologous Recombination (18, 21, 52, 73). CRISPR/Cas9 is a type II CRISPR system [reviewed by Makarova et al (51)], which is naturally present in many bacteria as an immunity mechanism to protect them against foreign DNA (27, 35). It consists of the Cas9 nuclease and a guide RNA (gRNA), a chimeric RNA molecule combining a CRISPR RNA (crRNA) and a transactivating RNA (tracrRNA), which together direct the Cas9 to cleave the target DNA sequence. This mechanism also requires the presence of a protospacer adjacent motif (PAM) upstream of the binding region (35). As a genome editing tool, CRISPR/Cas9 can be used to target virtually any genomic sequence next to a PAM site, by simply designing appropriate gRNAs. The generation of a double strand break (DSB) by the Cas9 induces cellular DNA repair mechanisms, like non-homologous ends joining (NHEJ), which is likely to introduce indels disrupting the targeted DNA sequence, or homology directed repair (HDR), if a donor construct is present. This system has been optimized for its application to human cells, including hPSCs, by transfecting a single or multiple vectors to co-express the Cas9 nuclease and the gRNAs (16, 18, 36, 52). As an alternative to the generation of a DSB by the wild type Cas9 enzyme, a mutated nickase version has also been developed, to facilitate HDR and reduce off-target mutations (18). Delivery of the CRISPR/Cas9 components via lentiviruses (76, 91), or adenoviruses (50), has also shown a high efficiency in targeting the human genome. To further improve the flexibility and rapidity of genome editing in hPSCs, Danwei Huangfu's group developed the iCRISPR platform, consisting of hESCs lines with doxycycline-inducible Cas9 expression (indicated as iCas9) (29). Transfection of iCas9 cells with gRNAs, derived via in vitro transcription, lead to over 40% efficiency for single gene targeting or around 10% for triple gene targeting.

Genome editing technologies are evolving fast and, as previously discussed, they represent a valid alternative to the use of iPSCs, because of the reduced variability associated with the use of isogenic cell lines. Alternatively, CRISPR/Cas9 could also be used to correct a specific genetic mutation in patient-derived cells. In this case, the rescue of the phenotype would provide the definitive proof of the connection between disease and genotype. When investigating the effects of CNVs including several genes, the creation of isogenic models would be excessively time consuming, but it should at least be considered to confirm the causality link between the gene(s) of interest within a CNV and the phenotype observed in differentiated iPSCs. In conclusion, the careful planning of in vitro modeling experiments and parallel use of engineered hESCs and iPSCs, allows the generation of very

elegant systems for the investigation of the cellular pathology underlying complex neurodevelopmental disorders.

## CONCLUDING REMARKS

Patient iPSC-based disease modeling has in a short time already shed light on the cellular pathology and molecular basis of neuropsychiatric disorders. This experimental model holds great promise despite the limitations discussed. We anticipate that CRISPR/CAS9 mediated genome editing of disease risk alleles in hPSCs will become a popular alternative in the disease modeling field, especially for diseases with monogenic mutations. For CNVs involving multiple genes, genome editing provides a means to demonstrate causality underpinning phenotype. Fully harnessing the potential of PSC-based disease modeling will benefit from the development of network models that contain different neuronal cell types (eg, cortical projection neurons and interneurons) and nonneuronal cells (eg, glial), and the improvement of high throughput culture systems for efficient drug screening.

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