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Modeling autism spectrum disorders with human neurons

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Abstract

Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disorders characterized by impaired social communication and interactions and by restricted and repetitive behaviors. Although ASD is suspected to have a heritable or sporadic genetic basis, its underlying etiology and pathogenesis are not well understood. Therefore, viable human neurons and glial cells produced using induced pluripotent stem cells (iPSC) to reprogram cells from individuals affected with ASD provide an unprecedented opportunity to elucidate the pathophysiology of these disorders, providing novel insights regarding ASD and a potential platform to develop and test therapeutic compounds. Herein, we discuss the state of art with regards to ASD modeling, including limitations of this technology, as well as potential future directions.

Keywords

autism spectrum disorders; disease modeling; human induced pluripotent stem cells; human neurons

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1. Introduction

Autism spectrum disorder (ASD) is a lifelong neurodevelopmental disability with a complex etiology, generally diagnosed based on criteria that include deficits in social communication and social interaction, as well as restricted, repetitive patterns of behavior, interests, or activities. Typical signs and symptoms are usually manifest in the early developmental period, although social deficits or behavior are often not apparent until later, when the child has difficulty meeting social or educational demands. In the United States, ASD is diagnosed in children as young as 53 months (regardless of gender or ethnicity). The prevalence is approximately 1:68 children, affecting four times more males than females (Wingate et al., 2014). Both early and long-term interventions are recommended; although those interventions can reduce symptoms of autism in children, responses are quite variable among individuals (Pierce et al., 2011; Warren et al., 2011), suggesting that better diagnostic tools are needed. Although the exact etiology of ASD remains unknown, a genetic component is likely (Geschwind, 2013; State and Levitt, 2011). Therefore, identifying genetic signatures and biological markers could facilitate diagnosis of autism in young children (Courchesne et al., 2015).

There are two categories of ASD, namely monogenic autism (due to a mutated gene) and complex/multigenic or idiopathic autism (uncertain genetic background). Monogenic forms of ASD include the following distinct genetic disorders: Fragile X syndrome, Rett syndrome, Timothy syndrome, Tuberous sclerosis, Joubert's syndrome, Angelman syndrome, and Phelan-McDermid syndrome (each accounts for no more than 1% of all ASD cases, with the entire group accounting for approximately 10% (Abrahams and Geschwind, 2008; Freitag et al., 2010; Geschwind, 2008). Therefore, most ASD individuals are idiopathic, with evidence of *de novo* mutations (especially for simplex families or hereditary mutations), or inheritance of common polymorphisms contributing to autism risk in multiplex families (Abrahams and Geschwind, 2008; Iossifov et al., 2014; Jiang et al., 2013; O'Roak et al., 2012). There are many chromosomal loci and genetic alterations implicated in ASD pathophysiology, consistent with the inherent heterogeneity of the disease (Geschwind, 2013). Complex ASD seems to be a combination of several genetic abnormalities that cause pathway damage (Geschwind, 2008). Consequently, for the vast majority of ASD cases, understanding pathogenetic mechanisms underlying ASD phenotypic behavior remains a challenge. Some mutations are related to synapse-associated molecules (Südhof, 2008), whereas for some other cases, perhaps there is an imbalance among excitatory/inhibitory neuronal circuitry (Mariani et al., 2015; Rubenstein, 2010). Notwithstanding, pathogenic mechanisms underlying autistic behavior remain unknown for the majority of ASD individuals.

Given the inherent heterogeneity of the genetic background associated with autism, modeling this disease using transgenic animals is inherently difficult. Brain samples collected postmortem from individuals with ASD have long been used to help clarify an autistic phenotype; however, that approach has important limitations, because usually the brain represents terminal stage of the disease; brain cells are dead and the tissue is fixed. Alternatively, an interesting strategy to study disorders that affect central nervous system (CNS) physiology would be to use developments in the burgeoning field of stem cells to

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produce target cell types for each disease. Using a pluripotent cell, e.g. embryonic stem cells (ESC), it is possible to produce, theoretically, any cell *in vitro*, including multiple functional neural cell types. Producing pluripotent cells from somatic cells (termed induced pluripotent stem cells, iPSC) has the potential to generate relevant cell types from genetic disorders. Fortunately, recent advances in cellular reprogramming (Takahashi and Yamanaka, 2006; Takahashi et al., 2007) have provided a breakthrough in human cellular disease modeling, making it possible to recapitulate live brain cells *in vitro*, while preserving the genetic background of individuals. The use of iPSC to generate viable human neurons or other neural cells *in vitro* has provided an outstanding opportunity to study a simplified neuronal network from a neurological disease with human genetic disease background preserved, which is particularly important for complex or multifactorial diseases like ASD (Beltrão-Braga et al., 2013; Marchetto et al., 2011; Marchetto et al., 2010; Mitne-Neto et al., 2011). Moreover, iPSC facilitates characterization of early developmental time points, giving information potentially useful in early diagnosis (including potential biological markers) and is particularly advantageous for understanding disease development and progress by IPSC-derived organoids (Mariani et al., 2015). These developments, in conjunction with exome and genome-wide sequencing data, would help to elucidate the neurodevelopmental course of autistic phenotypes (Willsey et al., 2013).

This review describes recent efforts related to ASD disease modeling using iPSC as stem cell source for *in vitro* production of neural cells. Based on findings summarized herein, it is clear that ASD disease modeling is already contributing to our understanding of disease etiology. Furthermore, this technology provides an unprecedented opportunity to manipulate ASD neural networks in a controlled environment to test strategies to recover altered neural phenotypes. In addition, these findings could also help us to better understand other neurodevelopmental diseases.

2. Disease Modeling

Since iPSC were first described, it has been used to model many diseases (Soldner and Jaenisch, 2012). For neurological diseases, where the raw material is often difficult to access, the use of iPSC to generate neurons (or other neural types) is particularly exciting. The first work to generate neural cells from iPSC was done using cells from a patient with amyotrophic lateral sclerosis (ALS) (Dimos et al., 2008). Although ALS-iPSC were successfully differentiated into motor neurons, cellular phenotype was not described. The first comparison between affected and non-affected cells derived from iPSC was a study published the following year, from a patient with spinal muscular atrophy (SMA). In this study, motor neurons derived from SMA-iPSC patient had low survival compared to motor neurons derived from a non-affected family member (Ebert et al., 2009). Nevertheless, since this first report, many others have been published, giving insights into unprecedented opportunities to study neurological diseases, including novel opportunities to test potential drugs to ameliorate or cure the condition.

Although almost any disorder can be modeled by iPSC, the challenge is identification of a robust and replicable cellular phenotype that is relevant to the target disease; unfortunately, this may be very difficult to achieve (Chailangkarn et al., 2012; Tiscornia et al., 2011).

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Several, neurodevelopmental disorders are popular targets for disease modeling using iPSCs, include Cockayne syndrome and ASD-related disorders, such as Rett syndrome (RTT), Fragile X syndrome (FXS) and even complex autism to test rare variants (de Sousa Andrade et al., 2012; Griesi-Oliveira et al., 2014; Marchetto et al., 2010; Urbach et al., 2010). Regardless, modeling complex autism is of particular interest, as the genetic background of each individual is preserved and any route involved in pathophysiology of autism could be investigated.

3. Monogenic Autism Disease Modeling

Monogenic autisms are neurodevelopmental disorders, usually with monogenetic causes identified, and whose individuals display clear autistic behaviors. It is noteworthy that some have already been modelled *in vitro* using iPSC technology (Amenduni et al., 2011; Marchetto et al., 2010; Pa ca et al., 2011; Urbach et al., 2010). Below and on table 1 we summarize the main findings of these reports.

Fragile X syndrome

Fragile X syndrome (FXS) is characterized by a trinucleotide repeat (CGG) expansion (> 200 times) on the 5′ fragile X mental retardation1 gene (*FMR1*), which leads to hypermethylation and gene silencing (Verkerk et al., 1991). The pathophysiology of FXS results in individuals with intellectual disability and a range of behavioral phenotypes (varying according to the number of trinucleotide repeats; (Rogers et al., 2001). In the first study based on iPSC-FXS modeling, there were epigenetic differences on *FMR1* gene expression; lines with gene silencing resulted in abnormal neuronal differentiation (Sheridan et al., 2011). In the two most recent studies involving iPSC-FXS modeling, there were neurons with reduced neurite length, fewer synaptic puncta and protein level and altered calcium influxes (Doers et al., 2014; Hagerman and Hagerman, 2013).

Rett and *MECP2* duplication syndrome

Rett syndrome (RTT) is a monogenic progressive neurological disorder caused by mutations on the X-linked gene methyl CpG-binding protein 2 (MeCP2; Amir et al., 1999). Unlike the majority of ASD individuals, RTT individuals are predominantly female, since affected males rarely survive or are severely affected (Villard et al., 2000). Symptoms RTT are very autism related, especially at the onset of disease, with loss of acquired motor language skills, and progressing to autistic behavior with stereotyped hand flaps, seizures, loss of speech and eventually leading to microcephaly, hypotonia and ataxia (Chahrour and Zoghbi, 2007; Percy, 2011). The *MECP2* gene is responsible to both activate and repress transcription (Chahrour et al., 2008), including actions in neurons, thereby acting as an important regulator with numerous targets (Skene et al., 2010).

In 2010, our group was the first to model RTT; we reported that iPSC-derived RTT neurons recapitulated many aspects previously identified in brain tissue recovered postmortem from a person with RTT, thereby providing credence to our model. In our study, RTT neurons had fewer synapses, reduced spine density, smaller soma size, altered calcium signaling, and electrophysiological defects when compared to controls (Marchetto et al., 2010).

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Interestingly, when modeling MECP2 duplication syndrome, we found neuronal phenotypes that go in opposite direction of what was observed in RTT-derived neurons (Nageshappa et al., 2015). This observation suggests that MeCP2 levels in human neurons must be tightly controlled. Furthermore, in preliminary studies to test the effect of some drugs on rescuing synaptic defects, insulin-like growth factor 1 (IGF-1) rescued RTT synaptic defects. In that regard, IGF-1 is a known neurotrophic factor (and is currently being used in clinical trials for RTT therapy). Furthermore, abnormal astrocytes were recently generated from iPSC-RTT, implicating them in neuronal abnormalities (Williams et al, 2014). In addition, in that study, IGF-1 or GPE (an IGF-1 peptide) partially rescued morphological defects of iPSC-RTT-astrocytes.

Timothy Syndrome

Timothy Syndrome (TS) is a rare autosomal dominant neurodevelopmental disorder caused by a mutation in the *CACNA1C* gene, which encodes for the voltage dependent calcium channel CaV1.2, leading to malfunction of this channel, causing high intracellular calcium concentrations (Splawski et al., 2004). Symptoms associated with TS include developmental delay, autistic symptoms, and heart malformations (usually accompanied by arrhythmia). In a recent study (Pa ca et al., 2011), iPSC-TS derived neurons were generated and had defects in action potential firing and $[Ca^{+2}]_i$ signaling, resulting in ineffective neuronal communication. These imbalanced neurons produced an overabundance of tyrosine hydroxylase, the enzyme necessary to generate the catecholamines norepinephrine and dopamine, two neurotransmissors with a key role in sensorial neurons and social behavior. In additon, it was reported that roscovitine blocked the defective calcium channel, reducing enzyme acculumlation. That similar effects were not reported in transgenic mice reiterated that there are often limitations to animal models of human diseases.

Angelman and Prader-Willi syndromes

Angelman and Prader-Willi syndromes (AS and PWS) are nerodevelopmental disorders associated with genomic imprinting, both caused by the same chromosomal deletion, on chromosomal region 15q11-13 (Knoll et al., 1989). Although AS and PWS have discrete phenotypes, they share neurological symptoms such as cognitive, social, and speech disabilities (Thibert et al., 2013; Whittington and Holland, 2010). In AS, deletion occurs on a maternal allele, reducing expression of ubiquitin-protein ligase E3A gene (*UBE3A*). In PWS, deletion occurs on the paternal allele, resulting in loss or reduction of expression of seven genes (Bittel and Butler, 2005). Although IPSC generated from both AS and PWS preserved DNA imprinting after reprogramming, unfortunately there were no apparent phenotypic differences among neurons derived from iPSC-AS, iPSC-PWS, and control neurons (Chamberlain et al., 2010).

Phelan-McDermid syndrome

Phelan-McDermid syndrome (PMDS) is a neurodevelopmental disorder caused by a deletion in the 22q13.3 region (Phelan and McDermid, 2012; Wilson et al., 2003), resulting in loss of genes, such as *SHANK3*, a protein in excitatory synapses that have been associated with autism (Durand et al., 2007). Symptoms include absent or delayed speech, intellectual disability, mental retardation, and autism. The iPSC-PMDS derived neurons had altered

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excitatory electrophysiology and fewer synapses. In addition, since these neurons had a deficit in *SHANK3* gene, these neuronal defects could be rescued either by using lentivirus to express SHANK3 or by exogenous IGF-1 (Shcheglovitov et al., 2013).

4. Complex/Multigenic Autism Disease Modeling

To investigate complex ASD, our laboratory generated an iPSC from individuals with classical autism, where syndromic forms of autism were excluded by genome sequencing. In several cases, the genetic analyses of their genome revealed one or more potentially causative mutations. As a proof-of-principle that iPSC modeling can be used to determine the contribution of individual genetic alteration, we choose one ASD subject carrying a *de novo* balanced translocation disrupting the *TRPC6* gene (Griesi-Oliveira et al., 2014), which encodes for the protein channel Transient Receptor Potential Canonical 6, a voltage-independent, Ca^{2+}- permeable cation channel. This gene has been implicated in neuronal processes known to be affected in ASD but was never implicated in ASD directly (Leuner et al., 2013; Zhou et al., 2008; Li et al., 2005). Furthermore, TRPC6 activates important neural development pathways, including the BDNF, CAMKIV, Akt and CREB signaling pathways (Li et al., 2005; Tai et al., 2008). Using iPSC, we investigated the functional consequences of this *TRPC6* haploinsufficiency. Neurons derived from TRPC6-mut iPSC had neuronal morphological and functional alterations compared to control neurons, such as altered morphology, including reduced total length and dendritic arborization. Key neuronal functions were also affected, including fewer dendritic spines and synapses, and impaired calcium dynamics (Griesi-Oliveira et al., 2014). It was noteworthy that some of these phenotypes were validated in mice. Neuronal phenotypes were rescued using candidate drugs, such as hyperforin and IGF-1. Hyperforin, a specific activator of TRPC6 channels, increased TRPC6 signaling. As mentioned previously, IGF-1 rescued neuronal defects in RTT-iPSC neurons (Marchetto et al., 2010). While it seems clear that this patient has other important genetic alterations, the investigation was able to show the relevant contribution of *TRPC6* loss of function to ASD.

Interestingly, we also observed that MeCP2 could control *TRPC6* expression. RTT-derived neurons have altered expression of TRPC6 and the MeCP2 protein occupies the *TRPC6* gene promoter, determined by chromatin immunoprecipitation. This apparent interaction reveals possible common pathways affected in syndromic and complex ASD. The study improved our understanding of ASD, as we demonstrated that an iPSC model of complex ASD had striking neuronal phenotypes, providing the basis for a potential drug-screening platform using human neurons as readouts.

Recently, another group (Mariani et al., 2015) used iPSC strategy to investigate neurodevelopmental alterations, specifically using three-dimensional neural cultures organoids derived from complex ASD-iPSC patients. Although genomic-wide investigation did not provide insights regarding the ASD phenotypic profile, based on transcriptome analyses, genes related to cell proliferation and neuronal differentiation were widely expressed. Interestingly, iPSC and neuro-progenitor cells (NPC) derived from ASD individuals did not have a higher cell proliferation rate than controls, but when more mature organoids were investigated in terms of proliferating cells, ASD organoids did not have

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decreased cell-cycle length. Moreover, synaptic assemble was also upregulated. Quantification of inhibitory VGAT (vesicular GABA transporter) by puncta counting revealed a significant increase when compared to excitatory VGLUT1 (vesicular glutamate transporter 1), suggesting an imbalance between inhibitory and excitatory synapses in neurons derived from ASD-iPSC. Also, GABAergic progenitor cells and neurotransmitter GABA were increased in organoids derived from ASD-iPSC. Transcriptome analyses of ASD-organoids revealed that FOXG1 could be responsible for overproduction of GABAergic neuronal lineage, and thus be a precursor for ASD (Mariani et al., 2015). In addition, as ASD individuals used in this study had macrocephaly, authors suggested that FOXG1 could be related to modulation of brain size, since patients with loss of function mutation in FOXG1 in patients with atypical Rett syndrome consistently had a small brain (Ariani et al., 2008; Bahi-Buisson et al., 2010; Mencarelli et al., 2010). Despite using only a small cohort of ASD individuals in this study, it was suggested that FOXG1 could be used as a biomarker for ASD severity, since macrocephaly is correlated with an adverse outcome. Finally, interference in FOXG1 expression restored normal density of GABAergic neurons (Mariani et al., 2015).

Although these data made important contributions to the elucidation of complex ASD, the number of ASD individuals studied was very limited, reinforcing the need for more lines from complex ASD individuals to generated and used to to validate common phenotypes and affected pathways, and to create robust diagnostic tools.

5. Future directions and take home message

The iPSC-disease modeling strategy, generating mini-brains (Lancaster and Knoblich, 2014; Lancaster et al., 2013) of individuals with neurological disorders, represents a novel and complementary strategy in ASD research and treatment (Mariani et al., 2015) (Nageshappa et al., 2015). However, current iPSC strategy has several important considerations and limitations, including clonal variability inherent in experimental methods, the use of appropriate controls and validation of specific cellular and molecular phenotypes in relation to the relevance of the ASD. Thus, many more iPSC models of complex autism are necessary to identify phenotypes and molecular pathways common to ASD. Ideally, clustering ASD individuals based on well-defined clinical parameters will be valuable during the validation process. However, to reach statistical significance, it may be necessary to work with hundreds of ASD individuals. In this direction, we have started working with several outreach programs to facilitate community engagement and sample collection, such as the Tooth Fairy Project. The latter, initiated in 2009, currently includes a website, a Facebook page and e-mail communications that connects to families and instructs them to send baby teeth from autistic individuals to our labs, from which dental pulp cells can be extracted and iPSC generated. Finally, we contend that comprehensive molecular and functional characterization of these iPSC-derived will be essential for reliable discovery of relevant ASD phenotypes that are driving ASD etiology. It is noteworthy that these molecular pathways should be targeted in future clinical trials.

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Highlights

- A new human model to study autism spectrum disorders
- Advances on modeling monogenetic autisms
- Efforts on modeling rare variants related to autism
- The use of induced pluripotent stem cells derived neurons for drug screening

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Table 1

Autism Spectrum Disorders modeled by iPSC.

	Prevalence	Mutated Gene	Relevant Cellular Phenotype	Rescue/Treatment	Reference
Fragile X Syndrome	1:4.000– 6.000	FMR1(X)	No	No	(Urbach et al., 2010)
			Fewer and shorter neuron's branches	No	(Sheridan et al., 2011)
			In neurons: reduced synaptic density, reduced neurite length, abnormal calcium transient and glutamate response	No	(Liu et al., 2012)
			Neurite outgrowth defect	No	(Doers et al., 2014)
Rett Syndrome	1:10.000	MeCP2(X)	In neurons: reduced soma size, dendritic spine density and synapses, calcium signaling and electrophysiological activity	Yes/ IGF-1 and gentamicin	(Maria C.N. Marchetto et al., 2010)
			Reduced neuronal soma size	No	(Cheung et al., 2011)
			Downregulated expression of neuronal marker	No	(Kim et al., 2011)
			Reduced Neuronal nucleus	No	(Ananiev et al., 2011)
			Astrocyte influence on neuronal morphology	Yes/IGF-1 and GPE	(Williams et al., 2014)
Timothy syndrome	20 cases reported	CACNA1C (12)	Defect in calcium signaling and neuronal electrophysiology, SATB2 expression, TH and catecholamine increased expression	Yes/ Roscovitine	(Sergiu P. Pasca et al., 2011)
			Dendritic retraction calcium-dependent	Yes/C3 transferase	(Krey et al., 2013)
CDKL5- related disorder	80 cases reported	CDKL5 (X)	No	No	(Urbach et al., 2010)
Angelman Syndrome	1:12.000	Maternal UBE3A (15)	No	No	(Chamberlain et al., 2010)
Prader- Willi syndrome	1:15.000	Paternal 15q11 – q13 (15)	No	No	(Chamberlain et al., 2010)
Phelan- McDermid syndrome	Over 600 cases reported	Shank3 (22)	Fewer synapses and impaired of excitatory neurotransmitter	Yes, IGF1	(Shcheglovitov et al., 2013)
Complex autism		TRPC6 (11)	Altered neuronal morphology, fewer synapses and fewer dendritic spines	Yes/IGF-1, hyperforin	(Urbach et al., 2010)
		FOXG1 (14)	Macrocephaly	No	(Urbach et al., 2010)