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Cellular Reprogramming: a novel tool for investigating autism spectrum disorders

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairment in reciprocal social interaction, communication, and the manifestation of stereotyped behaviors. Despite much effort, ASDs are not yet fully understood. Advanced genetics and genomics technologies have recently identified novel ASD genes. Approaches using genetically engineered murine models or postmortem human brain have facilitated understanding ASD. Reprogramming somatic cells into induced pluripotent stem cells (iPSCs) provides unprecedented opportunities in generating human disease models. Here, we present an overview of applying iPSCs in developing cellular models for understanding ASD. We will also discuss the future perspectives in use of iPSC as a platform to screen small molecules alleviating ASD and as a source of cell therapy.

Keywords

induced pluripotent stem cells; neurodevelopmental disorder; reprogramming; disease modeling; autism spectrum disorder; Rett syndrome; fragile X syndrome; Timothy syndrome

Introduction

Autism is a neurodevelopmental disorder defined by abnormal development of stereotypic behaviors, communication skills, and social interactions. The disease usually develops in early childhood and affects a child's learning abilities and emotions for the rest of their life. Brain developmental disorders causing autism are collectively called autism spectrum disorder (ASD). ASD includes Asperger syndrome and pervasive developmental disorder not otherwise specified (PDD-NOS). ASD places a considerable socioeconomic burden on affected individuals, their families, and society at large. In the 1990s, the prevalence of ASD was estimated to be less than 10 individuals per 10,000 in the United States [1], but recent data indicates a current prevalence of 110 per 10,000, affecting approximately 673,000 children [2].

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ASD has a strong genetic basis, as indicated by twin studies that have demonstrated a 70–90% concordance rate for monozygotic twins, several-fold higher than for dizygotic twins [3, 4]. Furthermore, siblings of ASD patients have a higher risk of developing neurodevelopmental diseases [5]. A small number of ASDs include well-defined monogenic syndromic disorders, such as fragile X syndrome, Rett syndrome, and Timothy syndrome. Recent advancement in genetics and genomics technologies has found novel ASDs genes that were not previously identifiable using traditional cytogenetic approaches (Figure 1) [6]. Despite much effort, there are no available cures for this disabling disease. ASD patients are clinically managed through education or behavioral treatments to improve social behaviors and decrease maladaptive behaviors [7]. The functions of putative ASD genes have been probed using murine models as well as post-mortem brains [8–10]. However, generating ASD murine models is not always feasible, and some neocortical areas involved in ASD are only present in human [11]. Furthermore, both normal and ASD post-mortem brains are not readily available for study. To overcome these difficulties, alternatives systems using pluripotent stem cells have been explored as easily accessible models of ASDs. In this review, we discuss the current status and future perspectives for studying ASDs using induced pluripotent stem cells (iPSCs).

iPSCs as a cellular model system for ASDs

Embryonic stem cells (ESCs) are derived from the inner cell mass of the blastocyst [12, 13], and because they are pluripotent can give rise to all three germ layers (Box 1). Due to the pluripotent and self-renewing capacities, ESCs are considered cellular resources for modeling human diseases as well as treating damaged human organs and tissues [14–17]. iPSCs have been created by reprogramming differentiated cells by the ectopic expression of the four transcription factors *Oct4*, *Sox2*, *c-Myc*, and *Klf-4* [18, 19]. iPSCs and ESCs share characteristics such as self-renewing capacity, morphology, expression of surface markers, and pluripotent differentiation potential. Because iPSCs retain the genetic composition of the parental somatic cells, the phenotypes of cells differentiated from iPSCs presumably represent those manifested by parental cells [20–25]. Thus, iPSCs from patients enable the development of disease-specific cellular models, platforms for drug screening, and autologous sources for cell replacement therapies. Furthermore, iPSCs bypass the ethical issues associated with destroying embryos to obtain human ESCs and immunological barriers that prevent the use of heterologous cells [26]. Figure 1 depicts a scheme in studying ASD using genomics, animal models and iPSCs. When a person is diagnosed by ASD, genomics method can identify the culprit candidate genes. The physiological functions of the genes can be examined by transgenic murine models [9]. Meanwhile, iPSCs are readily available by reprogramming somatic cells from ASD patients. Following neuronal differentiation, functional analysis can be performed to examine *in vitro* phenotypes manifested by ASD iPSCs. Quantifiable measures, such as neuronal connectivity, synaptic activity and neuronal migration that are associated with ASDs, can be used to test the efficacy of chemicals as a screening platform to ameliorate ASD.

Box 1

Definition of potency in stem cells

The ability of self-renewal and expanded differentiation potential define stem cells. Self-renewal is a form of proliferation without differentiation. Differentiation potential is classified into subtypes according to the **Potency** of stem cells. **Totipotency** is the ability of a single cell to produce all the types of cells necessary to give rise to an organism, including extraembryonic tissues. A zygote is a typical example of cells with totipotency. **Pluripotency** is the potential to differentiate into three germ layers: the endoderm, mesoderm, and ectoderm. Pluripotent stem cells, however, cannot generate

extraembryonic tissue or an entire organism. Embryonic stem cells (ESCs) are pluripotent stem cells. **Multipotency** is the ability to differentiate into multiple cell types, but only a limited number of closely related lineages. An example of multipotent stem cells is hematopoietic stem cells (HSCs). HSCs can give rise to all the cells in blood lineage, but not into other lineages, such as neurons or bone. **Oligopotency** is the potential to differentiate into a few cell types. Lymphoid and myeloid stem cells are typical examples of oligopotent cells. **Unipotency** is the limited ability to differentiate into only one cell type. Spermatogonial stem cells (SSCs) can self-renew but only differentiate into sperm.

When using iPSCs as ASD cellular model, a proper selection of standard control determines the interpretation of the *in vitro* phenotypes. Thus far, when iPSCs are used in cellular models, iPSCs from healthy persons have been used as controls for most disease models [21]. Because individual genetic variations have a large influence on cellular physiology, use of iPSCs derived from closely genetically related persons, such as siblings or parent, can reduce the compounding genetic effect. Nevertheless, isogenic iPSC lines are the ideal control. One method for generating isogenic lines is to take advantage of the fact that when a disease is X-linked and prominent in females, reprogramming produces unique sets of isogenic female iPSCs by retaining the active/repressed X chromosome status of fibroblasts [27]. Thus, iPSCs are produced with either the wild type or mutant allele on the active X chromosome from female Rett syndrome patients by taking advantage of this feature (see below and Box 2) [28–31]. However, recent study showed that inactive X chromosome in female iPSCs undergo erosion of X chromosome inactivation over time in culture, raising a concern in modeling X linked disorders using iPSCs [32]. As gene editing technologies such as zinc finger nuclease (ZFN) or Transcription activator-like effector nuclease (TALEN) are advanced, manipulating single gene within iPSCs has become possible (Box 3) [22, 33]. Gene-editing technology will become an attractive approach for generating isogenic controls or correcting mutated genes, when modeling ASDs.

Box 2

X chromosome status in human iPSCs and its relevance in ASD study

Female has two X chromosomes, one of which undergoes random inactivation during early embryonic development. Female somatic cells are mosaic in the expression of genes on X chromosome. Pluripotent ICM cells and thus the *in vitro* counterpart ESCs have cellular machinery to maintain two active X chromosomes. Murine iPSCs have two active X chromosomes as ESCs. However, X chromosome status of human ESCs is not as definite as murine ESCs. Human ESCs are considered derived from epiblast cells that have undergone random X chromosome inactivation, and have one active X chromosome. Despite a success in isolating human ESCs with two active X chromosomes, X chromosomes in human ESCs is not stable and subject to random inactivation by physiological stress [93, 94]. Thus, most female human ESCs have one active X chromosome. Reprogramming of human female somatic cells results in iPSCs that have one active X chromosome [27]. While most groups report the retention of parental X chromosome status [27, 30, 31], some reports showed the isolation of iPSCs with two active X chromosomes [50, 53]. Whether the erosion of inactive X chromosome of iPSC during long-term culture is solely responsible [32], or reprogramming results in X reactivation should be determined by further investigation. Nonetheless, the generation of mono-allelic iPSC line having one active X chromosome offers a unique opportunity to obtain isogenic controls for X-linked diseases. A handful of genes on X chromosomes were shown linked to ASDs, including NLGN4X, NLGN3, and RPL10 in addition to RTT and FX [95, 96]. Reprogramming fibroblasts of unaffected carrier females that have

cells expressing either wild type or mutant form of these genes will result in pure wild type or mutant iPSCs for direct comparative analysis as cellular model.

Box 3

Gene editing by ZFN and TALEN

Zinc finger nucleases (ZFNs) are modular enzymes generated by linking DNA cleavage domain to specific DNA binding domain to induce double strand break (DSB) in specific DNA sequence [97]. FokI endonuclease is used as DNA cleavage domain. Dimerization of FokI is required for the nuclease activity, and thus a pair of ZFN is designed to cleave target DNA. A zinc finger used for ZFN is comprised of a combination of cysteine and histidine residues, which recognize 3 bp of DNA sequence. DNA binding domain of ZFN is designed to target specific DNA sequence. The number of zinc finger repeats gives the specificity of ZFN. Once double strand break in target DNA sequence is generated, it is repaired by intrinsic DNA repair mechanisms such as non-homologous end joining (NHEJ) or homology directed repair (HDR). Insertions or mutations at the cleavage site accompany the NHEJ repair pathway, which disrupt the locus of interest during repairing process. Meanwhile, targeted insertion can be introduced into DSB sites by HDR, when provided with a homologous donor DNA. Targeted insertion allows for gene correction by homology-based approach. Although the ZFNs have been successfully introduced to genome editing in various organisms including human pluripotent stem cell (hPSC) [33, 98], nuclease specificity should be improved to prevent off-target cleavage that may cause toxicity in target cells. In addition, repairing DSB can result in unintended mutations and abnormal integration of donor DNA.

Transcription activator like effector nucleases (TALENs) provides an alternative approach for gene editing of specific locus. TALENs are another restriction enzymes generated by combining the TAL effector DNA binding domain with DNA cleavage domain [99]. In general, FokI endonuclease is used to make DSB in target site like in ZFNs. TAL effector proteins consist of repetitive 33–35 amino acid sequences that are highly conserved except for two amino acids of 12th and 13th. Those two key amino acids determine the specificity of TALEN binding to target DNA sequence [100]. TALENs showed much less off target effects and similar gene targeting efficiency, compared with ZFNs [101, 102]. Improving tools to deliver TALENs into target and to select the modified cells is needed in applying TALEN in iPSCs.

A robust neuronal differentiation method is critical in studying ASDs *in vitro*. Particularly, success in generating cortical neurons is essential, because potential mechanisms underlying autism include the defect in cortical connectivity and neural migration to cerebral cortex [34, 35]. There are two major types of neurons in cerebral cortex; excitatory glutamatergic projection neurons, and inhibitory GABAergic interneurons. Excitatory neurons are produced within cortical epithelium, while inhibitory interneurons are generated in the subpallium and migrate into dorsal telencephalon [36]. There have been much efforts to produce cortical excitatory neurons from pluripotent stem cells (Table 1). Broadly, neuronal differentiation is initiated either by forming embryoid body (EB) aggregates or by plating cells as adherent layer. Using SFEBq (serum-free EB-like quick aggregate) method, Sasai and colleagues have succeeded in generating cortical progenitors with regional specification by modulating Wnt3a and BMP pathways [37]. In adherent culture, Studer and colleagues expedited the neuronal differentiation and obtained Pax6+ neuronal progenitors within a week by inhibiting BMP and Nodal signaling [38]. Although Studer and colleagues did not extend the efforts to isolate forebrain neurons, Livesey and colleagues further refined the

approach to enrich the cortical excitatory neurons by using retinoids in addition to BMP and Nodal inhibitors [39]. The method developed by the Livesey group has not been used for any ASD study yet, but will be important in delineating the phenotypes critical in cortical neurons for ASD cellular models.

Genomics approaches to identify ASD-related genes

Identifying ASD-related genes has been challenging due to the phenotypic heterogeneity of ASD and the insufficient number of participants in association studies. However, collective efforts such as the International Molecular Genetic Study of Autism Consortium (IMGCA) and the Autism Genome Project Consortium (AGPC) have helped identify ASD genes [40, 41], as well as efforts using cytogenetic analysis, whole genome linkage studies, copy number variation (CNV) analysis, and whole genome association studies using array-based or massively parallel sequencing based genomics technologies [40–43]. In the near future, whole exome or whole genome sequencing will find very rare genes that contribute to ASD.

So far, variations in the chromosomal loci 15q11–13, 5p14.1, 16p11.2, 16q23.3, and 7q11.23 have been found in ASD patients. Included in these loci are the genes *NRXN1*, *NLGN3/4X*, *SHANK3*, *SHANK2*, *SYNGAP1*, and *DLGAP2* [44, 45], whose functions include proper establishment of synaptic structure, neuronal circuits, and synaptic homeostasis [44]. Previous analysis using animal models implicated the role of *NLGN3/4*, *SHANK3*, *Cntnap2* in synaptic network formation and social behaviors [46–48]. None of these candidate genes for ASDs were yet studied using human iPSCs. It would be important to find whether the neurons from these cells would recapitulate the phenotypes shown in murine model, and more importantly, human specific phenotypes. In the meantime, monogenic disorders that share symptoms with ASD, such as Fragile X syndrome (FX), Timothy syndrome (TS), tuberous sclerosis, and Rett syndrome (RTT), are being studied in the hopes of learning more about ASD etiology. Because these disorders are genetically well defined, studying these disorders in comparison with unaffected controls is likely to give invaluable insights into the causes of ASD symptoms. Thus, experiments using reprogrammed cells from patients affected by these monogenic diseases to study neurodevelopmental disorders are ongoing, as detailed in the next section (Table 2).

Monogenic syndromic ASDs

Rett syndrome

Rett syndrome (RTT) is an X-linked monogenic ASD. *De novo* mutations within methyl CpG-binding protein 2 (*MECP2*) gene are responsible for most cases of RTT. RTT patients exhibit clinical symptoms that include severe mental disabilities, absence of speech, stereotypic hand movements, encephalopathy, and respiratory dysfunction. The first RTT iPSCs were derived from a fibroblast cell line with a heterozygous Arg306Cys missense mutation in *MECP2* [49]. Full functional characterization of RTT iPSCs was carried out by Marchetto and colleagues [50] who derived iPSCs that express both wild type and mutant form of MeCP2 (Box 2). When directed to differentiate into neurons, random X inactivation occurred and neurons contained either wild type or mutant MeCP2, mimicking mosaic status of MeCP2 *in vivo*. Detailed molecular and functional examinations revealed that RTT neurons have fewer synapses, reduced spine density, smaller soma size, and reduced frequency of both calcium transients and spontaneous postsynaptic currents, which is consistent with those observed in post-mortem brain tissue of RTT patients and in animal models [50–52]. Furthermore, the authors rescued the RTT-specific phenotypes by expressing wild type *MECP2* or by treating mutant RTT neurons with IGF1 and gentamicin, showing the proof-of-principle in developing an *in vitro* platform for screening potential chemical therapies RTT. Several additional attempts were made to generate RTT iPSCs

from RTT patients [28–30]. In these studies, RTT iPSC lines that express either wild type or mutant MeCP2 were isolated and used as isogenic controls for comparison (Box 2). Cellular morphological phenotypes of RTT were confirmed in iPSC derived neurons [29, 30, 53]. Farra et al. extended the analysis of iPSC-derived neurons from murine RTT model [54]. They generated glutamatergic neurons from iPSCs derived wild type and RTT mouse, and found that MeCP2 deficient neurons display fewer action potentials, decreased action potential amplitude and peak inward current. The availability of widely accepted animal models for a given ASD, as exemplified in RTT, will be valuable to assess the iPSC-based model before clinical trials. Recent studies showed the non-neuronal pathogenesis of RTT mediated by astrocytes and microglia [55, 56]. Because iPSCs can be readily differentiated into glia cells, the non-neuronal function of MeCP2 can be investigated using RTT iPSCs. Furthermore, hematopoietic stem cells derived from isogenic wild type MeCP2 could be a novel opportunity for RTT cell therapy [56].

Fragile X syndrome

Fragile X syndrome (FX) is a X-linked dominant disorder associated with intellectual and emotional disabilities that range from mental retardation to autism. FX results from inactivation of the fragile X mental retardation gene 1 (*FMR1*) due to the expansion of CGG repeats in the 5' UTR region. CGG repeats cause transcription silencing of *FMR1* by enhancing methylation of the promoter as well as inhibiting translation of *FMR1* gene [57]. Human ESCs from a putative FX patient were derived after preimplantation genetic diagnosis (PGD) confirmed the presence of a pathogenic number of CGG expansions in *FMR1* [58]. When these FX-ESCs were differentiated, increased methylation of *FMR1* and a concomitant decrease of expression were observed, providing evidence for the critical role of epigenetic regulation of *FMR1* in FX pathogenesis [59, 60]. Recently, reprogramming cells from FX patients was attempted to model FX in vitro. Because factor-based reprogramming changes the epigenetic status of somatic cells, the expression and epigenetic status of *FMR1* was extensively studied in iPSCs [61]. Interestingly, FX-iPSCs retained the hypermethylation status of *FMR1* observed in fibroblasts, and thus did not express *FMR1*. This prevented the modeling of neuronal differentiation dependent silencing of *FMR1* [61]. Nonetheless, neuronal differentiation of FX-iPSCs would allow probing the function of *FMR1* in neurons. Indeed, another group isolated FX-iPSCs with 700 full mutation CGG repeats that retained the fibroblasts methylation status and did not express *FMR1* [60]. When neuronally differentiated, FX-iPSCs demonstrated the defect in neuronal differentiation, mimicking the FX phenotypes due to the mis-regulation of *FMR1* expression [60]. Since the mGlu5 inhibitors showed the recovery of phenotypes in murine FX models, testing the efficacy of mGlu5 inhibitors in FX-iPSC models will be an immediate test to validity of FX-iPSC models [62]. These will provide a platform to screen chemicals to rescue the FX.

Timothy syndrome

Timothy syndrome (TS) is an autosomal dominant disorder, and is caused by a mutation in the *cacna1c* gene encoding the calcium channel $\text{Ca}_v1.2$ subunit. The mutation in *cacna1c* results in delayed calcium channel closing, decreased channel inactivation, and increased cellular activation. Patients with TS suffer from dysfunctions in multiple systems, including cardiac arrhythmia, ASD and global developmental delay. Cortical neuronal precursor cells (NPCs) and neurons have been generated from iPSCs derived from individuals with TS [63]. TS neurons showed wider action potential compared with control neurons, and a defect in intracellular calcium signaling, consistent with the loss of channel inactivation in TS. TS mutation also caused a decrease in numbers of neurons with lower cortical layer markers but increase in those with upper cortical layers, suggesting a neuronal differentiation defect in TS. It would be interesting to examine the role of $\text{Ca}_v1.2$ in regulating cortical development,

because $\text{Ca}_v1.2$ has not been associated with cortical development before. Furthermore, TS mutation increased the proportion of neurons expressing tyrosine hydroxylase (TH), and thus caused the increase in the production of catecholamines, including dopamine and norepinephrin. The increase of TH production was reversible, and was blocked by treating neurons with an atypical L-type-channel blocker roscovitine as it rescued the abnormal action potential in cardiac model of TS [64]. These findings implicate the future development of roscovitine or related drugs in treating patients with TS or similar affected ASDs.

Schizophrenia

Recently, research on schizophrenia indicates that it is a neurodevelopmental disorder. Childhood-onset schizophrenia (COSZ) is defined as schizophrenia with an onset of psychosis before the age of 13 years, and is considered a severe form of schizophrenia. COSZ is preceded by and comorbid with ASD in 30% to 50% of cases [65]. Epidemiologic studies have demonstrated a connection between ASD and COSZ. COSZ has a strong genetic component with an estimated heritability of 80% to 85% [66], and a growing number of genetic abnormalities are shared by these two neurological disorders [67, 68]. Difficulty in modeling schizophrenia has impeded the understanding of these devastating diseases. Brennand and colleagues have used iPSCs an approach for elucidating the molecular mechanism of COSZ [69]. They used tetracycline-inducible lentiviral vectors encoding five reprogramming factors (OCT4, SOX2, c-MYC, KLF4, and LIN28) to derive iPSCs from four schizophrenia patients, one of whom was diagnosed with COSZ, and the others from families in which one parent and all of their siblings were affected with psychiatric disease. It was observed that the schizophrenia iPSC-derived neurons had reduced neuronal connectivity, reduced neurite outgrowths from somas, reduced PSD95 dendritic protein levels, and altered gene expression profiles relative to controls. Gene expression analysis implicated altered expression of glutamate, cAMP and WNT signaling pathways in the observed phenotypes. Interestingly, treatment with the antipsychotic loxapine improved the defects in neuronal connectivity and gene expression, providing a platform for screening antipsychotic drugs. Other groups independently confirmed the feasibility of use of iPSCs to model schizophrenia [70, 71]. Despite the consistent finding of lower neuronal network in schizophrenia iPSC neurons, Brennand et al did not find a defect in synaptic function possibly due to a relative small sample size. Extending the analysis in iPSCs using a large cohort of schizophrenia and COSZ patients will allow finding the common pathways leading to schizophrenia and those shared with ASDs [69].

Future perspectives on iPSC-based ASD modeling

As exemplified in above section, many studies already have shown the feasibility of using iPSCs as a cellular disease model of ASD [28, 61, 63, 69]. Despite the initial success in proof of principle studies, routine usages of iPSCs for ASD models still require improvement in procedures of iPSC-based cellular models (Figure 1). Most studies in monogenic syndromic ASD modeling used iPSCs generated by retroviral or lentiviral methods. The overwhelming influence of mutations responsible for phenotypes of ASD may have overshadowed the compounding effect on neuronal differentiation of iPSCs compared with hESCs [72]. However, previous study showed that lentivirus-mediated iPSCs produce less expandable neural progenitor cells due to the residual expression of reprogramming genes, which induces p53 pathways [73]. Alternative reprogramming methods, such as use of non-integrating viral vectors, non-integrating episomal vector transfection, use of excisable piggyBac transposons, repeated transient transfections, or the use of miRNAs and even proteins should be considered to minimize the influence of residual ectopic reprogramming proteins [74–77]. Regardless of reprogramming methods, inter-clonal variation among iPSC clones is one of the major issues in applying iPSCs for cellular

disease modeling [72]. Bock et al. has devised a unique system that scores the lineage specific differentiation potential of each iPSC by correlating the differentiation potential with the status of DNA methylation and lineage specific gene expression in undifferentiated state [78]. Thus, when a given iPSC line is to be used for neuronal differentiation in ASD modeling, performing global methylation and gene expression analysis will predict their differentiation potential and facilitate selecting iPSC clones that do not have bias in differentiation potential.

iPSCs have been produced from various human tissues, including fibroblasts, neuronal stem cells, peripheral blood, endothelial cells, hepatocytes, and keratinocytes [79–82]. Although iPSCs show the pluripotency regardless of donor cells, iPSCs retain epigenetic memory of donor cells. Epigenetic memory skews the differentiation potential toward the cellular lineage of the parental cells [83, 84]. When cultured continuously, the influence of epigenetic memory in iPSCs seems attenuated [83]. Thus, using iPSCs following the extended passage will minimize the altered differentiation capacity. However, long-term culture of human iPSCs and ESCs results in recurrent chromosome abnormalities, such as trisomy 8 or trisomy 12, that could affect the validity of *in vitro* disease models and safety of cell therapy [85, 86]. It is highly recommended to perform a regular quality control in iPSCs for *in vitro* and *in vivo* application.

In vitro neuronal differentiation from iPSCs is time-consuming multi-step procedures that involve production of neuronal progenitors, neuronal differentiation, and synaptically connected neurons, which takes over 2 to 3 months. Although laborious, the step-wise neuronal differentiation can be used to assess whether candidate ASD genes are involved in neuronal differentiation as shown in FX cellular models. In addition, multiple types of neurons generated from *in vitro* differentiation allow the unbiased assessment of function of ASD genes in a variety of cell types. However, the clonal variation can manifest the *in vitro* phenotypes undesired, and will require testing multiple iPSC lines in multiple neuronal differentiation. In order to complement the iPSC-mediated neuronal differentiation, alternative approach was devised that directly converts non-neuronal cells into neuronal cells without iPSC intermediate. The Wernig group used *Ascl1*, *Brn2*, and *Myt1l* to generate functional neurons (iN, induced neuron) from murine fibroblasts, and from human fibroblasts with additional *NeuroD1* [87, 88]. iN cells generated action potentials and formed functional synapses following over 6 weeks of neuronal induction. Because generation of iN cells does not require reprogramming step, it will largely reduce the efforts and time to derive and characterize iPSCs. However, iN cells are not expandable as iPSCs, and repetitive production of iN cells will be needed for each assay. Recently, induced neuronal stem cells (iNSCs) were generated using defined factors [89–91]. They are highly proliferative, and display morphological and molecular features of NSCs, producing neurons, astrocytes and oligodendrocytes upon differentiation. Human iNSCs have not been reported yet, but are expected to complement iPSCs and iN cells in modeling ASDs.

Closing remarks

Since iPSC generation was first described, there has been remarkable progress made in improving reprogramming technology and applying iPSCs to model a series of genetic and complex disorders. In this review, we have summarized the utility of iPSCs for studying ASD. Building current ASD models from tissues of genetically well-defined disorders, such as Fragile X syndrome, Rett syndrome, and Timothy syndrome, may provide appropriate cellular models for elucidating the pathogenesis of ASD, as well as the basis to establish high throughput screening platforms for the identification of novel drug interventions and cellular resources for potential *in vivo* cell therapy. High-throughput screening of potential therapeutic compounds will also benefit from a robust procedure to produce populations of

neuroepithelial-like stem cells from both hESCs and iPSCs [92]. Together, these recent advancements in cellular reprogramming provide the necessary tools to study human disease using relevant human cell types ‘in the dish’. This in turn will provide a platform to interrogate libraries of pharmaceutical interventions to help abrogate the disease phenotype.

Because iPSC technology is still in its infancy, many barriers remain to be overcome before it is widely adopted for clinical applications. These include optimization and standardization of protocols for reprogramming and differentiation into desired cells with increased efficiency, reduced variability between iPSC lines, and removing the possibility of tumor formation. More importantly, the characterization of patient-derived iPSCs, iPSC-derived functional neurons and glia should be rigorously evaluated before clinical use. Although it may take some years to address the drawbacks of this technology, iPSC-based applications have already proven their worth and, indeed, may hold the key to identifying and developing effective therapeutic interventions for ASD.

Glossary

Concordance	The occurrence of a same trait in both members of a pair of twins
X chromosome inactivation	A process by which one of X chromosomes in female becomes inactivated to compensate gene dosage differences between XX females and XY males
X chromosome reactivation	A process by which inactivated X chromosome by X chromosome inactivation is reverted to active state again, which is observed in cells of inner cell mass of early blastocysts and primordial germ cells
Prevalence	The total number of disease occurrence in the population for a given time period
Blastocyst	A thin walled hollow structure that is formed after the morula stage in early embryo development
Inner cell mass	A cluster of cells existing in the mammalian blastocyst those give rise to the embryo

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Figure 1. Overview of adopting induced pluripotent stem cell (iPSC) technology in autism spectrum disorder (ASD) study

ASD is a genetic neurodevelopmental disorder. Monogenic syndromic ASD is caused by well-defined genetic defect, including Rett syndrome, Fragile X syndrome, or Timothy syndrome. Advanced genomics tools are used to actively seek novel ASD genes. From skin biopsy or blood samples, patient-derived ASD-iPSCs can be generated. Cortical neurons that are most relevant neurons for ASD can be directed from iPSCs using well-established protocols (Table 1). The neuronal cells can be used to elucidate novel disease pathogenesis and to screen drugs as potential treatments. Recently developed gene editing technologies facilitate correcting the given mutation to provide isogenic control for *in vitro* diseases modeling or cell-based therapy.

Table 1

Strategies for differentiating human pluripotent stem cells into cortical neurons

Initiation	Strategy	Features	Ref.
Embryoid body (EBs) aggregate	SFEBq (serum-free EB, quick aggregate): continuous differentiation as EBs in the presence of DKK-1 and SB431542	Self-organizing structure forms cortical layer-specific neurons	[37]
	Formation of neural progenitors in EBs, plating to form rosette, and spontaneous neuron differentiation	Endogenous Wnt-mediated dorsal forebrain neuron formation	[103]
Adherent culture	High density adherent culture with Noggin, and SB431542	Inhibition of SMAD signaling induces rapid and complete neural differentiation	[38]
	High density adherent culture with Noggin, SB431542, and retinoids	Addition of retinoids directs cerebral cortical progenitors with high efficiency	[39]

Table 2

Disease models for autism spectrum disorders using iPSCs

Diseases	Genetic mutation	Neuronal population primarily affected	Genetics of inheritance	Differentiation	Functional outcome	Refs.
Rett syndrome	<i>MeCP2</i> mutation <i>CDKL5</i> mutation	Abnormal neuron maturation	X-linked dominant Most affected are female	TuJ ⁺ , GFAP ⁺ MAP2 ⁺ , VGLUT ⁺	Fewer synapses, reduced spine density, smaller soma size, altered calcium signaling electrophysiological defects, Reduced formation of mature neuron	[28, 50, 104]
Timothy syndrome	<i>CACNA1C</i> mutation	Axon growth, Synapse formation, Dendrite maturation	Autosomal dominant	MAP2 ⁺ , VGLUT ⁺	Defect in Ca ²⁺ signaling Abnormal norepinephrine and dopamine production	[63]
Fragile X syndrome	<i>FMR1</i> /CGG triplet repeat expansion	Global	X-linked dominant	TuJ ⁺ , GFAP ⁺	Silencing of <i>FMR1</i> transcription in FX-iPSCs Reduced formation of TuJ ⁺ neuron, Increase in numbers of GFAP ⁺ cells	[60, 61]
Prader Willi syndrome	15q11-q13 paternal deletion	Hypothalamic neurons	Paternal imprinting	MAP2 ⁺ , III-tubulin ⁺ , GFAP ⁺	Reduced level of snoRNA in PWS-iPSCs	[105]
Angelman syndrome	15q11-q13 maternal deletion	Global	Maternal imprinting	MAP2 ⁺ , TuJ ⁺	Up-regulation of the UBE3A-ATS and loss of UBE3A Showed AMPA receptor function	[106]
Schizophr enia	Sporadic	Abnormal neural distribution	Autosomal dominant/recessive	MAP2 ⁺ , III-tubulin ⁺ , VGLUT1 ⁺ , GAD1/2 ⁺	Decreased neuronal connectivity, neuritis number, PSD95-protein levels and glutamate receptor expression. Altered cyclic AMP and WNT signaling pathway	[69]