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Stem cells and modeling of autism spectrum disorders

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Abstract

Human neurons, generated from reprogrammed somatic cells isolated from live patients, bring a new perspective on the understanding of Autism Spectrum Disorders (ASD). The new technology can nicely complement other models for basic research and the development of therapeutic compounds aiming to revert or ameliorate the condition. Here, we discuss recent advances on the use of stem cells and other models to study ASDs, as well as their limitations, implications and future perspectives.

Keywords

autism spectrum disorders; stem cells; induced pluripotent stem cells; animal model; Fragile X syndrome; Rett syndrome; Angelman syndrome; Timothy syndrome; genomic editing

Autism

Autism is a developmental disorder that affects the brain's normal development of social and communication skills, with symptoms appearing in the first three years of life (A.D.A.M. Medical Encyclopedia, 2005). Since many different etiologies can generate this same behavioral outcome, the many disorders with autistic features, such as classical autism, pervasive developmental disorder not otherwise specified (PDD-NOS, also called atypical autism), and Asperger's syndrome are grouped under Autism Spectrum Disorders (ASD) (Berg and Geschwind, 2012). The ASD group also comprises Rett Syndrome (RTT) and childhood disintegrative disorder (CDD – initially termed childhood schizophrenia). In these two conditions the child is born with what appears to be normal development, but around the age of 3 for RTT and 10 for CDD, the acquired skills learned are lost (for example, language and coordination), and the autistic features manifest. Importantly, RTT is primarily a mutation on the MeCP2 gene, which categorizes it as one of the few autistic disorders with a

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known genetic cause. Disorders such as fragile X (FX), Angelman, Prader-Willi and Timothy (TS) syndromes are caused by specific chromosomal aberrations that also present neurodevelopmental and speech delays that can result in an autistic phenotype. Although they are not grouped under the ASD, these disorders as well as schizophrenia, can be studied along with ASD to provide new insights about the development and networking in the nervous system of the autistic phenotype. The exact number of autistic children born worldwide is difficult to estimate, either because this number is increasing with the improvement and availability of the diagnosis or simply due to an increase in the rate of affected newborns (Hertz-Picciotto and Delwiche, 2009, King and Bearman, 2009). Although the prevalence rate is predicted to be 1 in 15,000 in the current Diagnostic and Statistical Manual (DSM-IV-TR) by the American Psychiatric Association (American Psychiatric Association, 2000), most research reports identify the prevalence to be much higher (Fombonne, 2005, Kogan, et al., 2009). In 2008 in the United States, the Center for Disease Control estimated that 1 in 88 live births result in ASD, an increase of 78% from 2002 to 2008. Interestingly, it has been found that among children born with ASD, boys are five times more prone to be affected than girls (1 in 54 for boys and 1 in 252 for girls) (Centers for Disease Control and Prevention, 2012). In 63% of children with ASD, diagnosis occurs during the first 3 years of age when intellectual disability is not observed, as described in 14 sites in the United States in 2008 (Centers for Disease Control and Prevention, 2012). Even with the increasing efforts to improve the identification of ASD, there is still no medical test to diagnose ASD, and families rely on specialized professionals to conduct psychological and behavioral evaluations. The advances in clinical diagnostics alongside new genetic discoveries suggest that the number of children with ASD could be highly underestimated. An increase in the identification of autistic children directly affects the cost involved in the caretaking of these children. Furthermore, the cost is mainly shouldered by the family since most communities are not prepared to meet ASD needs. The annual medical expenses per child can range from \$2,100 to \$11,200, and with medical interventions this cost can increase to an average of \$50,000 per year in the United States. Their more specialized education can cost up to \$13,000 per year. As these children mature into autistic adults, the majority of them, do not live independently (Bruder, et al., 2012). The need for better diagnosis and treatment of ASD is therefore a concern that is increasing not only among scientists and physicians, but from an economic standpoint as well (Kogan, et al., 2008).

The autistic brain

From the perspective of a neurodevelopmental scientist, a primary goal is to better understand the complexities of the human brain by examining its course of development. Ideally, the fate of a cell could be traced by placing specific color markers in live tissue, starting at the time of its generation from progenitors, until maturation. This would lead to the discovery of the cell defects based on their live phenotype, maturation dynamics, networking profile, and laminar distribution. If this information could be correlated with genetic and proteomic data, there would be substantial and highly provocative information available to use towards developing a cure. Tracing cells through development is impossible to carry out in humans, however; and most of the live imaging techniques available provide

only a fraction of the information needed to better evaluate brain disorders of developmental origin. A major challenge for studies of the autistic brain is the large spectrum of diseases that are classified as ASD, since they present so many differences on the phenotype of the disorder. For example, a child diagnosed with Asperger's syndrome most often demonstrates normal speech capacity, IQ score, and is able to live a normal and independent life. In contrast, males born with a defect in the MeCP2 gene are severely affected by the gene disruption and in the majority of cases, cannot walk or communicate, making these individuals completely dependent on the care of others. These children often die at a young age. When conducting studies on autistic patients, it is also important to understand that the outcome of the disease is largely affected by the specific type of mutation. Taken together, it is somewhat expected that an individual incapable of speaking, walking, or performing tasks individually would present with disparities in the brain when compared to an individual that can easily execute such tasks. However, since few studies have compared different autistic subgroups with each other, the most significant differences may yet be unknown (Lotspeich, et al., 2004, Yu, et al., 2011). Another issue involved in autistic brain research is that most live imaging brain techniques, such as magnetic resonance imaging (MRI), require the individual to remain still during the exam, which is a difficult task for children and more severely affected adults. Furthermore, a large number of patients must be included that meet Diagnostic and Statistical Manual-IV (DSM-IV) diagnostic criteria as assessed by a specialized clinician in order for results to be considered significant. Factors such as age and gender should also be taken into consideration when performing brain imaging studies, since both have been shown to affect brain size during development (Aylward, et al., 2002, Courchesne, et al., 2011); in regards to gender, there are also differences in verbal and spatial domains (Beacher, et al., 2012). Although the MRI procedures present the issues mentioned above, many studies have shown interesting results and provided many contributions to our understanding of the autistic brain. In 1988 Courchesne et al. first identified that the cerebellum is smaller in autism, suggesting a developmental hypoplasia (Courchesne, et al., 1988). However, there were no differences in pathways to the cerebellum and midbrain in autistic individuals versus controls (Hsu, et al., 1991). These cerebellum findings were later challenged by another group that was unable to find significant differences in this area using the same method (Piven, et al., 1992). In RTT, the group led by Courchesne identified that these patients have a global hypoplasia of the brain, and present a progressive cerebellar atrophy that increases with age (Murakami, et al., 1992). The differences found in RTT patients were significant. Later, MeCP2 was identified as one of the primary causes of RTT in these infants, and so knowledge about the type of mutation can indicate an even more significant brain difference. The importance of the correlation between genotype and phenotype can be demonstrated with from RTT studies performed as emphasized by another author (Carter, et al., 2008). Other structural disparities in the brains of autistic patients have been observed in the amygdala-hippocampal complex, which was found to be significantly smaller in size (Abell, et al., 1999, Saitoh, et al., 2001). Another large study, with conflicting results, shows the amygdala is enlarged in autistic children, difference not seen in teenagers. The same study found the hippocampus to be enlarged in all ages (Schumann, et al., 2004). In addition, a decrease in white matter (McAlonan, et al., 2005) and significant increase in gray matter (Ecker, et al., 2012) were identified in autistic versus control individuals. Many others specific brain regions were

accessed by live imaging for detailed review in other studies (Anagnostou and Taylor, 2011, Stigler, et al., 2011). In summary, the volumetric distinctions found in the brain anatomy of autistic patients suggest a cell population variability, and such changes can only be studied by taking closer looks at the post-mortem brain or through *in vitro* modeling, in parallel to genetic analysis. Neuropathology studies are common when evaluating the differences in cell morphology and distribution, in addition to live imaging techniques. Some findings in MRI correlate directly with post-mortem analysis, such as a weight (size) increase in the autistic brain versus control at early ages, showed initially by Kemper, et al. (Kemper and Bauman, 1998). In this study, abnormalities in the neocortex were not found, although the author describes the neurons to be more packed in 8 out of 9 brains analyzed, and the number of Purkinje cells in the cerebellum reduced. Bailey et al., 1998 (Bailey, et al., 1998) identified megalencephaly in six handicapped autistic patients. Following these first volumetric measurements of the brain, most studies have focused on the identification of cellular abnormalities, such as increased number of neurons (Courchesne, et al., 2011) and microglia density (Morgan, et al., 2012) in the prefrontal cortex. The increased microglia density raises the question as to what role of the immune system plays in autism, since these cells appear to be activated in postmortem brain tissue. However, the main issues regarding post-mortem analysis are similar to that of live imaging, such as the sample size, gender, age and heterogeneity of the disorder itself. All of this, added to the possible lack of information on the medical or drug use history of the individual's brain being studied and the differences in the methodology or statistical analysis used between research groups. A summary of findings on the autistic post mortem brain until 2004 can be found for further reference (Palmen, et al., 2004). With the use of blood samples, clinicians are able to investigate the presence of cytokines, recently reviewed by Goines and Ashwood (Goines and Ashwood, 2012), suggesting a link between the immune system and autism. Even from an intracellular perspective, mitochondrial dysfunction has been associated with ASD (Rossignol and Frye, 2012). The finding that the immune system and specific cellular organelles may be reacting to a form of brain developmental abnormality accentuates the complexity of the ASD, requiring the use of an even more specific approach based on cell modeling and DNA analysis.

Autism and genetics

Although the specific genetic mechanisms and behavior underlying ASD may be varied or unknown, mounting evidence suggests that genetic defects or alterations at the neuronal synapse, as well as disparities in spine density, soma size and calcium signaling may underlie the pathophysiology (Garber, 2007, Marchetto, et al., 2010, Sanders, et al., 2012). The strongest association of ASD is with X-linked genes (fragile X syndrome) (Volkmar, 2009). The interaction of genes encoding postsynaptic neuroligins (NLGN) with presynaptic beta-neurexins (NRXN) is involved in the formation of functional synapses, suggesting that defects in synaptogenesis may underlie the etiology (Callan, et al., 2012). Mutations in these genes also regulate dendritic and axonal arborization in response to changing developmental demands (Knight, et al., 2011). An X-chromosomal epigenetic model also showed that neurons harboring the stably-active, expanded allele have reduced postsynaptic density protein 95 (PSD95) protein expression, reduced synaptic puncta density and reduced neurite

length. Significantly, such neurons are also functionally abnormal, having calcium transients of higher amplitude and increased frequency in comparison to neurons harboring the normal-active allele (Liu, et al., 2012). Patients with 1p21.3 microdeletions, which harbor the mir137 microRNA, were also associated with ASD synaptic phenotypes (Willemsen, et al., 2011). Further investigations also showed that some significant neurological disorders are correlated with misregulation or mutations of H3K4 gene (Wynder, et al., 2010) or neuronal LINE-1 retrotransposition activity in RTT that may ultimately affect brain development (Muotri, et al., 2010). In adult hippocampal neurogenesis, single-nucleotide polymorphism (SNP) mutations in PTEN gene also indicate a role in the pathogenesis of abnormal social behaviors, such as deficiencies in social interaction (Amiri, et al., 2012). Since these findings were made, a host of defects in candidate genes that coordinate synaptic transmission have been implicated sporadically in ASD in families (Freitag, et al., 2010). A portion of the sporadic nature of ASD may be attributed to spontaneous mutations (*de novo*) and are not only necessarily inherited from parents (Mendelian). These observations may explain why the search for common shared mutations has yielded so many inconclusive results (Sanders, et al., 2012). Consequently, gene discovery promises to help elucidate the underlying pathophysiology of these syndromes and directed to eventually improve diagnosis, treatment, and prognosis of ASD. This strategy is highly motivated because less than 15% of diagnosed cases of autism have an underlying cause identified (Mendelsohn and Schaefer, 2008). The advent of high-throughput sequencing (HTS) of genetic data created the possibility to explore, with high precision and thorough coverage, a large volume of genetic information at one time. Different types of HTS technologies exists, such as those used for extracting genomic or mitochondrial DNA (RNA-seq), coding or non-coding RNAs (RNA-seq, smallRNA-seq, lincRNA-seq), DNA-binding protein regions (Chip-seq) or protein-RNA binding specificity (Clip-seq) (Sugimoto, et al., 2012). In one of the first attempts to use HTS to study the genetic etiology of ASDs were sequenced exomes of 20 parent-child trios. Analyzing those 60 exomes in total, 4 attractive candidate genes (FOXP1, GRIN2B, SCN1A and LAMC3) involved in neurotransmission were found to harbor functional *de novo* mutations in sporadic families with ASDs (O’Roak, et al., 2011). Another finding with HTS identified the importance of RBFOXq. Expression alterations in the RBFOX1 splicing network in primary human neural stem cells during differentiation regulate a wide range of alternative splicing events. Such alterations are implicated in neuronal development and maturation, including transcription factors, other splicing factors and synaptic proteins, and are associated not only with ASD but with other related neurodevelopmental diseases (Fogel, et al., 2012). In neurons derived from patient-specific induced pluripotent stem cells (iPSCs), it was suggested that transcription factors and chromatin modifiers, such as POU3F2 and ZNF804A, and genes coding for cell adhesion proteins, including NRXN1 and NLGN1, are also associated with ASD abnormalities. In addition, from the same study, a number of novel long non-coding RNA (lncRNAs) were found to undergo dramatic changes in expression, one of which is HOTAIRM1, a regulator of several HOXA genes during myelopoiesis (Lin, et al., 2011). ASD susceptibility also occurs in some rare genetic variation at nucleotide level in the metabotropic glutamate-receptor (mGluR) signaling pathway, involving TSC1, TSC2, and SHANK3 genes, as well as the gene HOMER1. It encodes a postsynaptic density-localized scaffolding protein that interacts with Shank3 to regulate mGluR activity, as a novel set of autism-risk genes

(Kelleher, et al., 2012) that were also reported in a US patent (Margulies, 07/07/2011). Although current efforts are moving toward the use of HTS to discover novel causative variants, the bulk of the *de novo* mutations identified to date have been small chromosomal deletions or duplications in the form of copy number variations (CNVs) measured by the genotyping of large numbers of individuals (Cook and Scherer, 2008, Sebat, et al., 2007). CNV was showed to have direct associations with cadherins and protocadherins, implicating it in the neuronal cell adhesion pathway, or in the ubiquitin-proteasome system, which regulates synaptic attributes such as neurotransmitter release, synaptic vesicle recycling in presynaptic terminals as well as dynamic changes in dendritic spines and postsynaptic density (Glessner, et al., 2009). Other studies identified that genes with rare CNV defects, including NRXN1, NLGN3/4X, SYNGAP1, DLGAP2 and SHANK2/3, interfere in neurodevelopmental pathways by affecting maturation and function of glutamatergic synapses that may be disrupted in ASD (Cook and Scherer, 2008, Pinto, et al., 2010).

Mouse model of autism: where behavior meets genetics

The etiology and clinical phenotype of ASD comprises interactions between genetic and environmental factors. In this context, mouse models have been developed to provide a method to identify candidate genes and chromosomal regions associated with autism. Furthermore, genetically altered mice offer an approach to study the biological basis underlying autistic phenotypes at different levels of analysis, from molecular abnormalities identified in vitro to systems physiology and behavior. Advantages of using mouse models include population homogeneity, control of environmental variables, shorter time between generations, and the possibility for pharmacological screening and genetic manipulation (Reiss, 2009). Despite limitations in identifying behavioral phenotypes and the lack of some homologous neocortical areas in mice, mouse models for fragile X, RTT and Angelman syndromes have proved useful in revealing aspects of the neuropathological basis of the autism phenotype (Rakic, 2009). These models have been used to study abnormalities in synaptic plasticity, alterations in signaling pathways, neurotransmission and brain anatomy in autism (Moy and Nadler, 2008).

Fragile X Syndrome (FX)

FX is the most common cause of X-linked intellectual disability (Lubs, et al., 2012). The phenotypes range from learning disability to mental retardation and autism. Several human physiological phenotypes of FX can be recapitulated by FMR1 (fragile X mental retardation protein)-knockout mice. The combination of macroorchidism (enlarged testicles) and mental retardation in the absence of gross pathological abnormalities of testes and brain are characteristic in both FX patients and knockout mice (The Dutch-Belgian Fragile X Consortium, 1994). Transgenic mice also exhibit increased sensitivity to auditory stimulation and higher susceptibility to audiogenic seizures, consistent with sensory hypersensitivity and increase prevalence of seizures in FX patients (Chen and Toth, 2001, Musumeci, et al., 2000). In humans, the loss of FMR1 activity leads to devastating breakdowns in normal social behavior. FX mice express some behavioral phenotypes associated with the human syndrome, such as increased social anxiety (Spencer, et al., 2005), hyper-activity and reduced social interaction (Mineur, et al., 2002). Alterations in the

behavioral phenotype of FMR1 knockout mice include difficulties in object recognition and spatial learning (Brennan, et al., 2006, D'Hooge, et al., 1997, Mineur, et al., 2002, Ventura, et al., 2004). However, different results and the relative mildness of the behavioral phenotype in transgenic mice pose the greatest problems for the validity of animal models. Recent efforts have been made to develop detailed behavioral analysis to characterize behavioral indications of social deficits (McNaughton, et al., 2008). The impaired social cognition is reported by Spencer et al. (Spencer, et al., 2011, Spencer, et al., 2005) who observed altered anxiety and social behavior in FMR1 knockout mice and relation between behavioral responses and genetic background. Other groups observed reduced social interaction (Mineur, et al., 2006) and both higher social and lower nonsocial anxiety (Liu and Smith, 2009). Qiu et al. (Qiu, et al., 2009) reported increased expressions of the FMR1 gene in wild-type mice brain after kindling model to develop epileptic seizure. The kindling model is described elsewhere (Sato, et al., 1990). The FMR1 knockout mice displayed an accelerated kindling progression with prolonged electrographic seizures (Qiu, et al., 2009). The authors could suppress the rate of seizure progression by specific inhibition of N-methyl-D-aspartic acid receptor (NMDAR) and mGluR5 receptor, suggesting that FMR1 is related to limbic epileptogenesis signaling downstream of glutamatergic receptors. Deficits in cortical LTP (long-term potentiation), and higher LTD (long-term depression) associated with visual deficits in object recognition and spatial learning may result from disrupted glutamatergic signaling (Nosyreva and Huber, 2006, Zhang, et al., 2009). In support of this idea, Dolen et al. developed a transgenic mouse with reduction of metabotropic glutamatergic receptor on the FMR1 null background and observed a rescue in many of the phenotypes observed in the FMR1 null mice, including audiogenic seizures, and spine density in pyramidal neurons (Dolen, et al., 2007). Similarly, mGluR5 inhibition rescue acoustic prepulse in FMR1 null mice (de Vrij, et al., 2008). Inhibition of FMR1 signaling also ameliorated locomotor skills and anxiety behavior. These studies implicate potential new therapeutic targets in FX therapy. On a cellular level, neurons from FX patients and a knockout mouse model both exhibit the same abnormal dendritic spine morphology, characterized by immature filopodia shape (Irwin, et al., 2000). However, although human FX neurons have higher spine density, this is rarely found in the animal model. Atypical spine morphology suggests that FMR1 may have a role in neurotransmission, synaptogenesis and neuronal plasticity.

Rett Syndrome (RTT)

One of the first attempts to generate an animal model for RTT was developed in male mouse embryonic stem cells and was unsuccessful (Tate, et al., 1996). The chimeric embryos expressing a mutant form of MeCP2 showed severe defects, highlighting the essential developmental function of MeCP2. The study is in agreement with the predominance of girls with RTT (Armstrong, 1997) and with more severe symptoms found in boys with the syndrome (Schanen and Francke, 1998, Villard, et al., 2000). Two different groups solved this problem by using Cre-loxP technology to remove exons 3 and 4 of MeCP2 gene (Chen, et al., 2001, Guy, et al., 2001). The mice were viable and fertile, and exhibited phenotypes that resemble some of the symptoms of RTT patients. These MeCP2 knockout mice showed overtly normal development for about the first month of life, followed by increasing neurological abnormalities, and had a shorter life span of 6 – 12 weeks. Different MeCP2

mutations or truncated sequences have been used to create animals with symptoms similar to those observed in humans (Jentarra, et al., 2010, Pelka, et al., 2006, Shahbazian, et al., 2002). Mice with a truncated MeCP2 protein had a similar onset of symptoms including tremors and seizures, hypo-activity, and increased anxiety (Shahbazian, et al., 2002). Therefore, RTT symptoms appear during the establishment and refinement of neural networks in early postnatal development. Dani et al., demonstrated that pyramidal neurons have less spontaneous activity, mainly due to an imbalance between inhibitory and excitatory cortical neurons shifted to favor inhibition over excitation (Dani, et al., 2005, Dani and Nelson, 2009). These data are accompanied by a reduction in excitatory postsynaptic currents and by the presence of fewer and weaker connections in MeCP2 knockout mice, suggesting that loss of MeCP2 function reduces excitatory synaptic connectivity, leading to synaptic plasticity deficits (Belichenko, et al., 2009, Dani and Nelson, 2009). The behavioral analysis of mutated or knockout MeCP2 mice showed a significant reduction in social approach, atypical nesting and home-cage activity with no motor skill deficits, and reduced social interaction with no effect on social recognition as assessed by the partition test (Moretti, et al., 2005, Moretti, et al., 2006). Notably, re-expression of MeCP2 gene in knockout adult mice could restore full MeCP2 function and rescue several abnormal phenotypes. Guy et al., conditionally silenced MeCP2 and elegantly demonstrated that MeCP2 function can be restored by delayed restoration of that gene, raising the idea that some characteristics of Rett syndrome are reversible in mice (Guy, et al., 2007). Using a similar approach, Lioy et al. crossed an induced Cre-recombinase transgene driven by the human astrocytic glial fibrillary acidic protein (GFAP) promoter with mice containing a Cre-excisable transcriptional stop codon sequence in the endogenous MeCP2 gene (Lioy, et al., 2011). The presence of functional MeCP2 protein in astrocytes generated phenotypic changes in mutant neurons in vivo, restoring dendritic morphology and the levels of glutamatergic markers. Biochemical and cellular rescue was also associated with improvement in motor skills, normalization of abnormal respiratory patterns, and a prolonged lifespan. Therefore, understanding the mechanisms by which different MeCP2 mutations lead to RTT may reveal effective strategies for improving the associated symptoms.

Angelman syndrome (AS)

AS is a severe neurological disorder caused by maternal deficiency of an imprinted chromosomal region 15q11-13, leading to loss of ubiquitin protein ligase E3A (UBE3A) (Veltman, et al., 2005). The knockout mice for the maternal UBE3A gene have abnormal motor coordination, substantial deficits in long-term potentiation, impaired learning and memory, and increased seizure susceptibility (DeLorey, et al., 1998, Homanics, et al., 1997, Jiang, et al., 1998, Miura, et al., 2002, van Woerden, et al., 2007). Additionally, using the visual cortex of the knockout mouse as a model, impairment was detected of excitatory neocortical plasticity during experience-dependent development (Yashiro, et al., 2009). The demonstration that the physiological substrates of synaptic plasticity remained intact raised the possibility that behavioral or pharmacological manipulations could improve brain function in individuals with AS. Another group observed diminished excitatory neurotransmission in neocortical pyramidal neurons due to an excitatory/inhibitory imbalance at cellular and circuit levels, which may contribute to seizure susceptibility

(Wallace, et al., 2012). Moreover, Van Woerden et al. (van Woerden, et al., 2007) reported rescue of certain molecular and cellular deficits by the introduction of an additional mutation at the inhibitory phosphorylation site of alpha-calcium/calmodulin-dependent protein kinase II, an enzyme related with hippocampal plasticity and learning (Elgersma, et al., 2002). These studies show that UBE3A plays an important role in experience-dependent plasticity and synapse development (Greer, et al., 2010, Yashiro, et al., 2009). The chromosomal region 15q11-13 also contains several GABAA receptor genes, including subunits B3, A5, and G3 (DeLorey, et al., 2008). GABRB3 knockout mice have high rates of neonatal mortality. Mice that survive have enhanced seizure susceptibility, abnormal motor coordination, and deficits in learning and memory (DeLorey, et al., 1998, DeLorey, et al., 2008, Homanics, et al., 1997). These mice also exhibit behavioral phenotypes associated with ASD such as impaired social interaction and hyper active with stereotyped circling behavior (DeLorey, et al., 2008).

The advent of Human Induced Pluripotent Stem Cells (iPSCs)

The human Embryonic Stem Cell (ESC) field emerged as a promising area in developmental sciences. For the first time, researchers have the possibility of exploiting early stages of human development *in vitro*. This promises to soon become one powerful tool for insights into human neurodevelopment (Marchetto, et al., 2011). The progress to understand neurodevelopmental diseases was hampered by the scarce availability of disease-specific human ESCs, carrying specific genetic alterations in the genome. For ASD, just one human ES cell line with a naturally occurring mutation has been obtained, after a preimplantation genetic diagnosis revealed the presence of the genetic condition predisposing to FX (Eiges, et al., 2007). The emerging ESC field also had to deal with unique ethical issues, given the nature of the samples. In 2006, Yamanaka's group successfully obtained embryonic-like stem cells from mouse embryonic fibroblasts, which present the hallmarks of the pluripotent counterparts including indefinitely self-renewing and the potential for differentiation to any cell type of the body (Takahashi and Yamanaka, 2006). The breakthrough uses a relative simple approach: a set of transcription factors to jump-start and reprogram all the genetic network landscape to a pluripotent stage, challenging the prevalent dogma that adult cells have limited flexibility. More than evading ethical issues, the new technique gained attention for the potential of generating disease-specific pluripotent stem cells with unprecedented simplicity. Since then, several iPSCs lines have being generated to diverse neurodevelopmental disorders. Given the uniqueness of human cognition and behavior, an *in vitro* human neurodevelopmental model able to recapitulate early stages of the development could pinpoint specific biochemical and cellular features of this species, difficult to reproduce in other models (Han, et al., 2011, Konopka, et al., 2012). The iPSC technology also makes possible the manipulation of these alterations with candidate drugs, seeking to rescue the found phenotypes and paving the way to future drug-screening platforms. Given the strong association of the genotype and phenotype in ASD, disorders of monogenic origin were first reprogrammed to iPSC. Several iPSCs from ASD were successfully generated and differentiated into neural cells.

FX

Given its high prevalence among the population, FX is the most common cause for ASD of monogenic origin (Kindler and Kreienkamp, 2012). CGG trinucleotide repeat expansion in the 5'UTR of the fragile X mental retardation 1 (FMR1) gene results in hypermethylation of its promoter and transcriptional silencing (Feng, et al., 1995). A study using a human ESC with pathogenic expansion of the repeat demonstrated that the FMR1 gene is unmethylated and expressed in an undifferentiated state, but epigenetic silencing occurs upon differentiation (Eiges, et al., 2007). However, when iPSC are generated from individuals carrying the FX mutation, the FMR1 gene still retain the epigenetic markers of silencing and no FMR1 protein was detected (Urbach, et al., 2010). Although this limits the use of FX-iPSC to model the silencing of FMR1 gene upon differentiation, they are still valuable tools to study the phenotype of FMR1 absence in neural population.

RTT

Recently, several works have independently described the generation and characterization of iPSC-derived neurons from RTT patients (Ananiev, et al., 2011, Cheung, et al., 2011, Hotta, et al., 2009, Kim, et al., 2011, Marchetto, et al., 2010). Together, these reports represent the most comprehensive set of iPSC research that has been conducted on a neurological disorder thus far. Our group first reported that the genome of a patient with RTT leads to a set of altered neuronal physiology, recapitulating many aspects of the disease in iPSC-derived neurons, such as reduced dendrite arborization, decreased spine density, diminished glutamatergic synaptic puncta and defective calcium influx (Marchetto, et al., 2010). In another work, a reduction in the soma and nuclear size was found in RTT neurons compared to controls (Cheung, et al., 2011). Different from our work, in other studies isogenic RTT and control iPSCs were derived through retained X-inactivation, representing a promising source of mutant and control cells (Ananiev, et al., 2011, Cheung, et al., 2011, Kim, et al., 2011). Finally, a defect in neuronal maturation was seen in RTT iPSC-derived neurons when studying the gene expression profile of some key neuronal genes during differentiation (Kim, et al., 2011). Aside the classical RTT, with mutations in the MeCP2 gene, several forms of atypical RTT (aRTT) also exist, where the MeCP2 isn't the culprit. Among these variations, iPSC lines have been generated to the Hanefeld form, which is caused by mutations in the CDKL5 gene (Amenduni, et al., 2011, Ricciardi, et al., 2012). One of the studies confirmed a synaptic phenotype, with CDKL5 mutant neurons harboring a reduced VGLUT1 and PSD-95 puncta together with abnormal spine morphology (Ricciardi, et al., 2012). These observations, interesting, are mirroring the ones already published with RTT iPSC. However, the synaptic rescue found previously (Marchetto, et al., 2010) still need to be confirmed in these aRTT neurons.

Timothy Syndrome (TS)

TS is a rare autosomal dominant disorder that has a spectrum of physical manifestations, including cardiac arrhythmias, heart malformations, syndactyly and autism. It is caused by mutations in the calcium channel known as CaV1.2, encoded by the *cacna1c* gene (Splawski, et al., 2004). In TS patients, these mutations lead to longer opening conformation time of the channel, resulting in an abnormal accumulation of intracellular calcium.

Depending on the cell type, this abnormal influx of calcium manifests distinct phenotypes. The high prevalence of ASD and cognitive impairment among individuals with TS indicate a crucial role of CaV1.2 and intracellular calcium homeostasis in the normal development of the nervous system (Splawski, et al., 2004). In an iPSC model of the disease, the authors confirmed a defect in differentiation together with several other neuronal abnormalities, when compared to control cell lines (Pasca, et al., 2011). Differentiating neuronal cells have defects in calcium signaling, increased expression of tyrosine hydroxylase (TH) and catecholamines, and decreased proportion of cells expressing lower cortical layers markers (Pasca, et al., 2011). The link between ASD phenotype seen in TS individuals and the observed cellular phenotype still needs to be clarified, but the increase in TH and catecholamines is in agreement with valproic acid models of ASD (D'Souza, et al., 2009). Additionally, the deficit in neuronal differentiation potential can be contributing to the cognitive impairment in patients. Finally, treatment of TS iPSC-derived neurons with the L-type channel blocker roscovitine promoted a reduction in the number of TH positive neurons, indicating that a drug screening of these neurons using calcium channels modulators can potentially find new ways to ameliorate TS condition.

Schizophrenia (SZ)

SZ is an impairing neurological disorder characterized by paranoia, hallucinations (both described as positive symptoms), and loss of affect, with feelings being split from outside world (considered a negative symptom). Even not being part of the ASD, diverse brain features affected in SZ overlaps with ASD, bringing hope that insights with this disorder will ultimately help to clarify also ASD. The disease onset is generally during late adolescence or early adulthood (Koenen, et al., 2009). A combination of several factors, including genetic and environmental, are considered to play a role in the SZ manifestation. In a recent publication, SZ iPSCs were generated from four patients with complex genetic mutations (Brennand and Gage, 2012). More than recapitulating phenotypes described from post-mortem studies, they observed specific neuronal phenotypes: reduced neuronal connectivity in conjugation with decreased PSD95 protein levels, neurite number and altered gene expression profiles compared to controls. Additionally, key cellular and molecular elements were improved after treatment of the SZ neurons with an antipsychotic drug, demonstrating the versatility of the iPSC system.

Altogether, these works represent the first steps on the ambitious promise of the iPSC technology in recapitulating neurodevelopmental disorders. The power of the iPSC system, allowing the study of different stages of the neural development, pointed to some interesting observations, specially at the synaptic levels, where the autistic cells seems to be defective when compared to controls. Most neurodevelopmental diseases are believed to have an onset after some time of development, with the individual being considered to have a normal phenotype until this changing point. Studying these ASD models, however, researchers revealed alterations in early stages of the neural development. This raises the question of when the disease really starts. The disease onset may represent a shift where the natural plasticity of the brain cannot sustain a minimal normal homeostasis anymore. In vivo validation of this observation may open several new therapeutical opportunities for mental disorders. The introduction of iPSC was a breakthrough in the study of human

neurodevelopmental and neurodegenerative diseases in controlled conditions. Using iPSC, researchers are beginning to have an idea of how to study developing neurons from patients, in a restless journey to find specific phenotypes. The “disease-in-a-dish” concept allows in vitro understanding of the underlying diseases mechanisms, screening for lead compounds that can revert disease-specific phenotypes, and long-term cellular transplantation to study the importance of the environment on the onset of the disease or for potential therapeutic use. However, identification of subtle abnormal phenotypes from background-related variations could prove difficult task to the lack of genetically matched controls. Different genetic background is a particularly significant limitation to in vitro disease modeling approaches, mainly due to variations in the cell derivation process (Lengner, 2010), mutations and residual effects of viral vectors introduced during cellular reprogramming (Hussein, et al., 2011, Soldner, et al., 2009), and disease penetrance and progression (Summers, 1996). For the “disease-in-a-dish” and “patient-specific medicine” to be truly successful, it is essential to generate isogenic cell lines that can specifically recapitulate phenotypes of monogenic syndromes.

Genomic editing

Several studies took advantage of the nonrandom pattern of X chromosome inactivation in female iPSC to generate isogenic pairs of wild type and mutant lines from RTT (Ananiev, et al., 2011, Wong, et al., 2012) and FX patients (Liu, et al., 2012). However, Mekhoubad et al. showed that erosion of the X chromosome in female iPSCs for modeling Lesch-Nyhan syndrome occurs over time in vitro, raising significant considerations for modeling X-linked disorders (Mekhoubad, et al., 2012). Alternatively, genome editing technology such as zinc finger nuclease (ZFN) or transcription activator-like effector nucleases (TALEN) can be used to manipulate the genome by exogenously introducing DNA fragments and correcting mutated genes via homologous recombination (Capecchi, 1989, Hockemeyer and Jaenisch, 2010, Lombardo, et al., 2007, Zou, et al., 2009). ZFN are modular enzymes designed to recognize a specific DNA sequence by combining zinc finger proteins and an endonuclease to induce double-strand breaks (Bibikova, et al., 2003). Since a dimerization of FokI endonuclease is necessary for its proper function, a pair of ZFN is formed to bind the target region in the opposite orientation in order to cleave the DNA (Porteus and Carroll, 2005). As a proof of principle, Zou et al. demonstrated the important role of ZFN in inducing target gene modifications in hESC and hiPSC, providing a solid basis for genomic editing in pluripotent cells (Zou, et al., 2009). Other studies illustrated how ZFN are a powerful tool to target genes that are not even expressed in hESCs (Hockemeyer, et al., 2009); and to induce chromosomal translocation at specific loci in human mesenchymal precursor and embryonic stem cells (Brunet, et al., 2009). Although several studies have reported that stem cells after ZFN-mediated targeting show no effect on cell karyotype and pluripotency after prolonged culture, zinc fingers may be further refined for higher specificity to better discriminate gene target and off-target binding sites. TALENs are the latest technology for gene editing of a specific locus. TALE transcription factors were originally discovered in plant pathogens to overcome host genome regulatory machinery (Kay, et al., 2007). TAL effector proteins consist of repetitive 33–35 amino acid sequences that are highly conserved except for the 12 and 13 aminoacids. The 12th and 13th amino acid residues in sequential repeats determine the DNA binding specificity and thereby the TAL effector’s target site. The simple

relationship between amino acids in the TAL effector and the DNA bases in its target provides a means of engineering TAL effector proteins with an affinity for a pre-determined DNA sequence. Similar to ZFN, TALENs are associated to endonuclease to create a double-strand break at a particular genomic site (Miller, et al., 2011). One important advantage of TALENs over ZFN is the cloning method. The “Golden Gate” cloning methodology allows a plasmid to be assembled from 10 separate input plasmids without the introduction of any mutation (Engler, et al., 2009). The efficiency of this cloning methodology was proved by Tong et al., (Tong, et al., 2012) in generating gene-specific knockout ESC Hockemeyer et al., (Hockemeyer, et al., 2011) and developing elegant construct architecture to introduce cassettes solely at the TALEN-specified location in hESC and hiPSC. The authors stated that the specific site was produced with similar efficiency and precision as do ZFN but with fewer off-target effects. New technologies to generate TALEN are being published at high rate further consolidating the power of this technology and giving hope to develop more efficient genomic editing tools (Cermak, et al., 2011, Mussolino, et al., 2011, Scholze and Boch, 2010).

Drug screening

Despite limitations regarding population heterogeneity and epigenetic changes, embryonic and induced stem cells make patient specific therapy a real possibility, while overcoming immune rejection in transplantation. Moreover, iPSC derived cell types allow in vitro study of drug toxicology, and can accelerate the never-ending search for new therapeutic agents (Bosnjak, 2012). High throughput drug screening platforms combined with a better knowledge of the signaling pathways that control stem cell fate and differentiation can increase the efficiency of various differentiation protocols, and facilitate therapeutic applications of stem cells (Ebert, et al., 2012). Classically, synthetic small molecules and natural compounds have provided useful chemical ligands to study complex cellular processes. Such molecules will likely provide new insights into stem cell biology, and may contribute to effective regenerative medicine (Ding and Schultz, 2005, Ding, et al., 2003). In 2006, Chen et al. (Chen, et al., 2006) used a library of 50,000 in a multi-well high-throughput platform to identify compounds that help to maintain the cell pluripotency in feeder free mouse ESC. Similarly, a high throughput assay was developed in order to discover new compounds capable to regulate hESC self-renewal and differentiation (Desbordes, et al., 2008). The author started with a 2880 compound library in 384 well plates to select 65 for further dose response analysis resulting in 22 confirmed hits to promote cellular proliferation. A selected set of four out of ten compounds was ultimately found to decrease the levels of OCT4, and further validated to induce differentiation inducer. These studies demonstrated how HTS can increase our understanding of hESC biology and be applied in drug development and cell therapy (Underhill and Bhatia, 2007). Until recently no studies have used a drug screening platform to select drugs that can rescue abnormal phenotypes of neurological diseases. For example, a lack of access to human motor neurons and appropriate disease models has hampered efforts to test new drug candidates for ALS. One recent study used ALS patient-specific neurons derived from iPSC to verify whether four histone acetyltransferase inhibitors can rescue ALS motor neuron phenotype (Egawa, et al., 2012). Even using a low throughput assay, the authors created the foundation for the development of new platforms aimed at the rescue of diseased phenotypes.

Future Perspectives

Because of the considerable clinical and genetic heterogeneity of the ASDs (Berg and Geschwind, 2012), it is not surprising that there are currently no approved treatments for their core symptoms. However, with further characterization of the genes and sequencing of genes in thousands of families, we will be able to develop novel therapeutics and preventive strategies for ASD. The HTS technology has proven to be useful for identification of functional mutations underlying the autism spectrum disorders, suggesting that HTS of larger numbers of samples becomes more commonplace, in a way that the genomic and transcriptomic landscape of rare mutations as well as proteomic data underlying ASDs will expand considerably. A challenging scenario would be one that involves combining different HTS technologies to correlate different levels of genetic information and understand the pathways involved. By analyzing the information of genomic DNA with the transcriptomic expression profile and epigenetics influence, one could create the possibility of generating proteomic data that leads to the understanding of ASD phenotype development. Studies involving HTS analysis are becoming more appreciable with the high availability of free bioinformatics tools and fast distributed computing architectures for biological data storing and analysis, giving sufficient resources for the accurate design of biological models. This resource would allow for the suggestion of specific genetic corrections in isolated genes or entire biological networks, and direct the path toward ASD treatment. Genetic tools, such as ZFNs or TALENs are examples of an alternative approach to gene editing at a specific locus, which could be utilized in ASD correction (Bibikova et al., 2003). However, as with any other type of therapy, determining the use of stem cells to be appropriate for cell therapy use is inevitably dependent on the level of safety, and whether any side effects on the tissue exist. Ultimately, if institutions combined their personal banks of iPSC and drug screening methods in a research consortium, the combination of different patients with improved diagnostics could lead toward a better drug treatment that would ameliorate the symptoms of this devastating disorder, and potentially direct us towards a cure. By characterizing various ASD phenotypes based on information taken from the molecular and genetic to the cognitive levels, data can be organized to thoroughly represent the various degrees of severity in a given ASD. For example, two factors imperative in determining the level of severity would be the amount and frequency of verbal and nonverbal communication observed in a patient. The use of iPSC technology specific to each classified category would then perhaps allow for more comprehensive understanding of the ASD, and ideally, better targeted drug screening. However, given the multi-faceted and complicated nature of ASD, this would require the collaboration and investment of various disciplines, from the level of molecular neuroscience to bioinformatics and cognitive psychology, in order to optimize the development of a treatment. From the perspective of a researcher, it is an immense challenge to strive in the direction of a treatment while maintaining balance between the development of specific expectations, and the creation of realistic goals. It is therefore important to understand that while great efforts are being invested and progress continues to grow in the field of iPSC technology many questions will continue to arise and give way to further investigation and improvement in our understanding and treatment of ASD.

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Figure 1.

iPSCs patient specific therapy. From an autistic subject it is possible to obtain an examination on their behavior, brain structure and network, together with genetics for an attempt to identify the type of autism. The postmortem brain gives us information basically on the brain structure and cell distribution. With a simple non-invasive method by obtaining the baby tooth from an autistic kids, it is possible to cultivate these dental pulp cells and reprogram them to an induced pluripotent state (iPSCs). From iPSC we are able now to differentiate them into neuronal stem cells (NSCs) and these can be further differentiated into a more specialized brain cell, like neuron or glial cells. From NSCs to mature brain cells we are able now to model the disease in a dish, filling the next pieces on the puzzle to better direct the prognostic of autism with the molecular insight along. From the cellular modeling we can obtain morphology, electrophysiology and proliferation. The molecular profiling of proteins and their pathways, in addition to genetic information obtained also from cell type modeling, all together these insights help to fill the puzzle of autism with specific drug treatments