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Induced pluripotent stem cells: a new tool to confront the challenge of neuropsychiatric disorders

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Abstract

Studies in the area of human brain development are critical as research on neurological and psychiatric disorders has advanced, revealing the origins of pathophysiology to be in the earliest developmental stages. Only with a more precise understanding of the genes and environments that influence the brain in these early stages can we address questions about the pathology, diagnosis, prevention and treatment of neuropsychiatric disorders of developmental origin, like autism, schizophrenia, and Tourette syndrome. A new approach for studying early developmental events is the use of induced pluripotent stem cells (iPSCs). These are cells with wide potential, similar to that of embryonic stem cells, derived from mature somatic cells. We review the protocols used to create iPSCs, including the most efficient and reliable reprogramming strategies available to date for generating iPSCs. In addition, we discuss how this new tool can be applied to neuropsychiatric research. The use of iPSCs can advance our understanding of how genes and gene products are dynamically involved in the formation of unique features of the human brain, and how aberrant genetic variation may interfere with its typical formation. The iPSC technology, if properly applied, can also address basic questions about neural differentiation such as how stem cells can be guided into general and specific neurodevelopmental pathways. Current work in neuropsychiatry with iPSCs derived from patients has focused on disorders with specific genetics deficits and those with less-defined origins; it has revealed previously unknown aspects of pathology and potential pharmacological interventions. These exciting advances based on the use of iPSCs hold promise for improving early diagnosis and, possibly, treatment of psychiatric disorders.

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Introduction

An emerging concept is that neuropsychiatric disorders arise from deviations from the regular differentiation programs of the CNS, leading to altered ratios of specific neuron types and disruptions in patterns of connectivity. In the majority of cases, this is caused by a combination of gene variants as well as environmental factors, although in rare cases a single gene variant of strong effect may be sufficient to cause altered development and thus illness. For example, changes in volume and number of specific cell types are observed in the cerebral cortex of patients with depressive disorders and in the basal ganglia of patients with Tourette syndrome (Kataoka et al., 2010; Peterson et al., 2001; Rajkowska et al., 1999). These abnormalities do not represent drastic departures from the regular program of development, rather, they possibly are the extremes in the range of individual variations in the programs that build the CNS. Neuroimaging studies have revealed structural and functional brain abnormalities in many neuropsychiatric conditions, often preceding the onset of symptoms (Tau and Peterson). For example, at the earliest ages assessed, children with attention deficit hyperactivity disorder have significantly less cerebral cortical volume than typically developing children (Castellanos et al., 2002). Even children and adolescents at risk for but without symptoms of major depressive disorder showed reductions in cortical thickness (Peterson et al., 2009) and retrospective studies have shown that patients with schizophrenia were more likely to have reduced head circumference at birth (Cannon et al., 2002). Thus, the earliest stages of brain development are implicated in the trajectories leading to the manifestation of neuropsychiatric disorders.

Understanding how typical developmental programs occur and how they may be modified in disease is of great importance for advancing our knowledge and improving our ability to diagnose and treat disorders of the CNS. Great strides have been made in our capacity to trace neural development in animals, particularly invertebrates, lower vertebrates, and small mammals like rodents. This work has been crucial for understanding fundamental laws of neural development that may be conserved throughout evolution; however, it is often difficult to use such animals as models for human disorders given the greater complexity of the human CNS and its behavioral manifestations. Unfortunately, research on primate and human neural development has been severely limited until recently by ethical issues, inaccessibility of the brain, and other problems.

A completely new perspective was opened by the isolation of human embryonic stem cells (ES cells) from blastocysts, in 1998 (Thomson et al., 1998). This work revealed the possibility of studying neural development in a human cellular system, albeit *in vitro*. However, there are only few available human ES cell lines, which are different from one another in terms of their potential, and there are concerns over the genetic stability of ES cells after long-term amplification *in vitro*. Moreover, the ethical debate over the destruction of human embryos has prohibited the generation of a large number of embryonic stem cell lines.

In 2006-2007, Yamanaka and colleagues showed that four transcription factors, Oct4, c-Myc, Sox2 and Klf4, were sufficient to reprogram mouse and human skin fibroblasts into stem cell-like cells, called induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007). A flurry of subsequent papers from multiple independent groups confirmed the feasibility of deriving iPSC from somatic cells using a different cocktail of factors, which included Nanog and Lin28 (Meissner et al., 2007; Wernig et al., 2007; Yu et al., 2007). Investigators determined that iPSCs were remarkably similar to embryo-derived stem cells with respect to gene expression profile, epigenetic marks, and fate potential (Mikkelsen et al., 2007). Although it is clear that iPSC are not identical to ES cells as they retain an epigenetic memory reflecting the tissue of origin (Kim et al., 2010; Polo et al., 2010), most iPSCs lines

pass the most stringent tests of pluripotency, self-renewal, multilineage potential and, for mouse iPSCs, germline transmission (the ability to generate most mouse tissues after injection into an early embryo, including germ cells) (Boland et al., 2009; Zhao et al., 2009). In addition, iPSCs, like ES cells, when injected in immunocompromised mice, are able to form teratomas containing derivatives from the three embryonic germ layers, a further proof of their pluripotency.

In these last few years, iPSCs have been a major focus of research. Despite significant efforts, their derivation is still inefficient (typically, using current techniques, less than 0.1% of fibroblasts become iPSCs) and the characterization of “truly” reprogrammed cells versus those that are only partially reprogrammed or unstable has proven difficult. Also, there are potentially serious risks in those iPSC generation procedures that introduce foreign DNA into cells (Saha and Jaenisch, 2009) that limit their use for therapeutic purposes. Procedures that do not obligatorily involve DNA integration are still at an early stage of evaluation (Seifinejad et al.) (Table 1), although the recent suggestion of using *in vitro* transcribed synthetic mRNA appears promising (Warren et al.). Despite the challenges, there are potentially many advantages of iPSCs over ES cells, as the former can be generated from a specific individual, maintaining his/her genetic constitution and identity. In addition, iPSCs represent a source of differentiated cell types genetically identical to the person of origin that may be useful for screening effective drugs for individual forms of pathology and for being a source of transplantable tissue.

Reprogramming strategies

There are two main problems with the reprogramming technology using viruses: first, the low efficiency of the process, because only about 0.02% of the transduced skin fibroblasts are turned into ES-like cells, and second, the random retroviral integration into the host-cell genome. Retroviral and lentiviral vectors are powerful tools for *in vitro* and *in vivo* gene transfer and also the most common choice for the expression of the pluripotency-inducing transgenes because of their relatively high transduction efficiency (Takahashi et al., 2007; Yu et al., 2007). However, use of viral vectors in somatic cell reprogramming could limit therapeutic use of the reprogrammed cells in human clinical settings, such as transplantation. In order to avoid random and stable genomic integration of foreign DNA sequences, alternative approaches such as transient transfection of plasmid DNA, electroporation of single multiprotein expression vectors, non-integrating episomal vectors, Cre-excisable polycistronic lentiviral vectors, non-integrating viruses such as adenoviruses and Sendai viruses, recombinant proteins and modified synthetic mRNA, have been employed to produce iPSCs, thus facilitating the future use of these cells. In addition, the *piggyBac* transposon system has been also used, a moth-derived DNA transposon which is active in mammalian cells and allows transposable DNA elements to be excised and inserted back into the genome without leaving a footprint after excision (Woltjen et al., 2009). Table 1 outlines different factors used to reprogram human somatic cells to pluripotency.

Transfection of plasmid DNA encoding for the four factors initially used by Yamanaka has been shown to induce reprogramming in both mouse (Okita et al., 2008) and human (Si-Tayeb et al., 2010) somatic cells. The simple transfection method using lipid-based transfection reagents showed that it is possible to reprogram human fibroblast without any DNA integration; however, reprogramming efficiency was 10 times lower than viral based reprogramming (one iPS cell from 300,000 fibroblasts) (Si-Tayeb et al., 2010). Also, using this approach, very sensitive cell colony screening with southern blot or PCR methods must be routinely employed, since it is possible to have unwanted DNA integration.

An alternative approach to reprogram human somatic cells involves the electroporation of a single reprogramming cassette (c-Myc, Klf-4, Oct4, Sox2) linked with 2A-peptides; this technique apparently yields a relatively high reprogramming efficiency (2.5%) (Kaji et al., 2009). Similarly, Yu et al. (2009) recently showed that electroporation of non-integrating and extrachromosomally replicated episomal plasmid vectors containing EBNA1/oriP and encoding the following genes: OCT4, SOX2, NANOG, KLF4, LIN28, c-MYC and SV40LT, could reprogram human somatic cells, albeit at low reprogramming efficiencies (~3 to 6 colonies/10⁶ input cells). Similar to plasmid DNA transfection which does not require any viral packaging, caution must be exercised if these cells are to be used in the context of human clinical therapy, because unwanted random integration of the plasmid vectors could occur during transfection and culture.

An elegant approach, first described by Rudolf Jaenisch and his group, involves reprogramming human somatic cells using a polycistronic reprogramming cassette delivered by a single viral vector followed by its removal mediated by Cre/LoxP recombination (Carey et al., 2009; Soldner et al., 2009). Although this approach successfully removes multiple transgene sequences, reprogramming efficiency was again very low (about 0.0001%) and this technique leaves in the modified cell residual vector sequences (i.e., a single 5' LTR sequence with LoxP site), which can still create insertional mutations. Another promising approach for somatic cell reprogramming was described by Andras Nagy's group and combined the single vector with a *piggyBac* transposon system (Woltjen et al., 2009). *PiggyBac* transposons requires one 5' and one 3' terminal repeat that flanks a transgene and also transient expression of the transposase enzyme in order to achieve insertion or excision of the transgene. The transposon-mediated reprogramming system enables complete elimination of exogenous reprogramming factors without genetic alteration. Although reprogramming efficiency is high, removing multiple transposons is labor intensive and requires another transient transfection step with a transposase-expressing plasmid.

Recently, non-integrating strategies for somatic cell reprogramming using replication-defective adenoviruses (Zhou and Freed, 2009) and Sendai virus (Fusaki et al., 2009; Seki et al., 2010) have been reported. Although this type of viral approaches generated human iPSCs without any viral integration, their production and handling requires virology knowledge and strict biosafety regulations. To create integration free iPSCs from mouse and human somatic cells, protein delivery-based approaches such as proteins fused with arginine-rich cell-penetrating peptides have also been developed (Kim et al., 2009; Zhou et al., 2009). For example, Kim et al. (2009) showed that human cells could be reprogrammed to iPSCs using the classic 4 reprogramming factors (Oct4, Sox2, Klf4 and c-Myc) fused with 9 arginine residues. The efficiency of iPSC generation (0.001%) was significantly lower than that of virus-based protocols, and this technique required more time (8 weeks) for the establishment of initial iPS colonies. Finally, more efficient and viral-free iPSCs generation from human somatic cells has been reported using *in vitro* transcribed modified mRNAs encoding the reprogramming factors (Warren et al., 2010). Although one obstacle of this elegant approach was daily transfection of modified mRNA for 16 days, the efficiency of iPSCs generation was two orders of magnitude higher than that typically reported for retroviral transduction protocols. Thus, among the various methods to generate patient-specific pluripotent cells by nonviral methods without disturbing the host genome, *in vitro* transcribed mRNA transfection protocols appears very promising.

Genetic considerations

As the techniques for generating human iPSCs are refined and these cells become a more widely used tool for understanding brain development, the insights they produce must be

understood in the context of the greater complexity of the human genome and the human brain. The characteristics that distinguish the recently evolved primate and human brain from other mammalian brains include the proportionally larger growth of the cerebral cortex, the diversification of cortical area maps and a much more extensive degree of connectivity (Rakic, 1995). It can be argued that these differences in scale and complexity have driven an increase in the size of neurons, a larger metabolic demand and an increased proportion of glial cells.

Another important aspect of the human brain is the high degree of morphological and functional variation from one individual to another. Similar variation is found in genomic sequence, when comparing individual human genomes (Kim et al., 2008; McCarroll et al., 2008). In addition to single nucleotide polymorphisms (SNPs), human genomes contain an abundance of copy number variation (CNV) and structural variation (SV) events. These consist of entire blocks of DNA sequence, ranging in size from less than 1 kb to several millions bp, that have been deleted, duplicated, inserted, translocated or inverted (Hurles et al., 2008; Korbel et al., 2007; Levy et al., 2007). The average number of such CNV/SV per individual was previously estimated between 700 and 1400 depending on the platform chosen for the experiment and the ethnicity of the subject (Conrad et al., 2010; Park et al., 2010). Recent results (Mills et al., 2011) show that individuals may have 2000 or more CNVs. Supplementary table 10 from the same article shows that CNVs overlap with more than 5500 genes, and change the structure or the function of the gene product for 2500 or more genes across different individuals. Thus, CNV/SVs may alter the coding potential of at least 5-10% of the known genes. The number and size of events that can be detected using high-throughput sequencing depends upon the coverage (or, number of reads per sample).

Although these CNV/SV are scattered all over the genome, “hot spots” have been identified, for example, an 8-megabase (Mb) region in chromosome 22q11.2 and an 18-Mb region at 7q11 (Korbel et al., 2007), among others. Interestingly, DNA sequence deletions in the 22q11.2 and 7q11 hot spot regions are associated with two developmental neuropsychiatric disorders, velocardiofacial syndrome (VCFS) and William-Beuren Syndrome (WBS), respectively. Patients with VCFS have a high frequency of learning disorders, autism spectrum disorders (ASD) and schizophrenia. Patients with WBS are characterized by developmental delay with pronounced peculiarities of language and emotional functioning.

Furthermore, an increasing number of studies link rare CNV/SV to neuropsychiatric disorders (Manolio et al., 2009). Rare CNV/SVs include deletions/duplications at 16p11.2, associated with ASD and idiopathic mental retardation, and deletions at 1q21.1, 15.q13.3 and 22.q11.2, found in schizophrenia (Sebat et al., 2007; Stefansson et al., 2008; Weiss et al., 2008). An increase in the occurrence of *de novo* large deletions has been reported in individuals with ASD (Christian et al., 2008; Sebat et al., 2007).

The large degree of interindividual variability makes it difficult to determine which of the multitude of genomic sequence variants carried by an individual is responsible for a given phenotype. Because of these challenges, current approaches focus on determining the functional consequences of the genomic variants at the transcript and biological levels. Recent studies suggest that gene transcripts expressed in the developing human brain encompass a much larger set of mRNA variants and splice patterns, not found at corresponding stages of animal brain development (Johnson et al., 2009). By paying attention to the biological consequences of genetic variants, which genetic variants should be pursued for diagnostic and treatment purposes may become more clear (Figure 1).

Investigating how human genetic variation leads to variation in structure/function of the human brain requires the ability to follow neural development at the cellular and systems

level. The derivation of iPSCs from skin or other differentiated somatic cells might permit study of how natural genetic variation affects neurodevelopment and how it produces individual differences in brain function and behavior. The success of this approach will depend on our ability to reconstruct brain development *in vitro* in a way that mimics the biological steps that enable an embryonic stem cell to differentiate into neurons and glia from specific regions of the CNS. If this is accomplished, we could, in principle, understand the molecular programs of development that may underpin normal and abnormal development (Figure 1).

Another question that could be approached using the iPSC technology is that of how, given the substantial similarity in gene number between mammalian species, the substantial differences in brain structure and function amongst mammals are encoded at the genomic level. It seems possible that differences in sequences of non-coding areas of the genome, and therefore in gene regulation, might be a crucial component of such differences.

Another important component to consider is epigenetics, i.e., the regulation of the chromatin due to histone modifications and the methylation of cytosines in the DNA. The development of iPSCs would enable us to study regulatory processes that establish the dynamic gene networks driving the differentiation of a particular cell type at a particular time, whether determined by DNA sequence variants, mRNA variants, or altered states of chromatin. Importantly, the environmental, hormonal and toxic effects on the differentiation dynamics and related gene expression trajectories can also be explored.

The decreasing costs and the increased availability of deep sequencing technology will allow in the near future a comprehensive examination of structural DNA variation, epigenomic changes, and gene expression. For example, deep sequencing of RNA and DNA isolated from neural cells differentiated from iPSCs, which in turn derived from a specific individual, may allow us to correlate common and rare individual genetic variation with patterns of gene expression and specific biological properties of these neural cells, i.e., proliferation, differentiation and survival, during the cellular processes of neurodevelopment (Figure 1). With the advancement of bioinformatics and the standardization of protocols used to generate and characterize iPSCs, we may be able to eventually understand the combined effects of multiple genetic variants on the cellular processes of neurodevelopment, and assess the impact of epigenetic effects and environmental variables on both gene expression and biological function. The efforts have already started with the 1000 genomes project (Durbin et al., 2010; Mills et al., 2011).

Regulation of neural differentiation

The iPS and ES technologies offer us a unique opportunity to study the fundamental, species-specific events that regulate neural differentiation. Overall, there is a large degree of similarity in both gene expression and epigenetic marks among iPS and ES cells. Recent work has shown that iPSCs harbor a “gene signature” that characterizes them regardless of their origin or method of derivation, suggesting that iPSCs represent a unique subtype of pluripotent cells (Chin et al., 2009). However, there is often incomplete silencing of fibroblast genes in iPSCs and failure to fully induce embryonic stem cell genes, likely reflecting incomplete resetting of somatic gene expression (Chin et al., 2009). Also, iPSCs retain an epigenetic memory of their tissue of origin that conditions their ability to differentiate into specific cell types (Kim et al., 2010). Yet, studies have shown that iPSC progressively becomes more similar to ES cells with repeated passaging. Highlighting the fundamental similarity in gene expression between iPS and ES cells, many fundamental tenets of developmental neurobiology were confirmed in both cell types; for example, that

forebrain phenotype arises by default, that retinoic acid treatment posteriorizes the neural tissue, and that glial cells differentiate later than neurons (Hu et al., 2010).

Current research efforts are focusing on the discovery of appropriate methods for directing the ES and iPSCs into the neural lineage, and for the subsequent derivation and growth of neuronal and glial cells specific from a region of the CNS. Several basic protocols currently exist, most of which have been applied to mouse and human embryonic stem cells. Most of the protocols are 2-dimensional cell culture systems (Cohen et al., 2007; Elkabetz and Studer, 2009) that involve an initial aggregation of the cells into embryoid bodies, followed by the generation of “neural rosettes”, aggregates of neural cells with a defined polarity similar to the in vivo CNS (Johnson et al., 2007; Pankratz et al., 2007). In most cases, the cells can be directed into different fates (forebrain, midbrain, spinal cord) by the use of specific morphogens and culture conditions. For example, motor neurons have been generated by treatment with retinoic acid (Li et al., 2005). However, these two-dimensional culture models do not recapitulate the specific layer structure, area patterning and connectivity that characterize the mammalian CNS. The possibility also exists of using 3-dimensional cell aggregates that maintain a neural tube-like structure (Eiraku et al., 2008). We have recently applied the *Eiraku et al* technique to the study of iPSC-derived neural stem cells differentiation and maturation with initially promising results (Figure 2). One of the most remarkable findings is that neurons derived from iPSCs display typical synapses and classic action potentials, which raises our hope to utilize these cells for understanding how “diseased” iPSCs are altered in functional aspects of the neural network. However, the efficiency of neural differentiation appears to be more variable in iPSC lines compared to ES cell lines. Some of this variability is likely to be caused by inter-individual differences in genetic background and variable epigenetic status among the different lines under investigation, i.e., residual epigenetic marks that stochastically affect different lines (Osafune et al., 2008). Future refinements of the de-differentiation and re-differentiation protocols may address these questions. This remains a major challenge for future studies.

The applications of iPSCs to pathological conditions

While there is great promise that iPSCs may allow us to understand typical development and the genes and cellular processes involved, another major goal of such research would be to apply that knowledge to understand and treat disease. Hence, the ability to use the iPSC technology to also examine diseases directly, with cells derived from patients and their family members, is of particular interest. For disorders of the nervous system, where the direct study of diseased tissue is limited by issues of accessibility and the inability of mature neurons to regenerate, the ability to study diseased neural cells derived from iPSCs may provide breakthroughs for diagnosis and treatment.

Disorders of the nervous system in which a genetic origin has been identified will allow immediate correlations to be made between cellular changes in development and functioning with disease. One example in which this potential of iPSCs has already been demonstrated is familial dysautonomia (FD), a severe disorder in which sensory and autonomic neurons degenerate, leading to the patient's early death. Patients with FD have a known mutation in the gene encoding I- κ -B kinase complex-associated protein (IKAP), resulting in lower levels of IKAP protein. Previous work using mouse models of related disorders and lymphocytes from patients had suggested that a cell migration defect at the early stages of the nervous system development was involved in the pathogenesis of FD (Close et al., 2006; Iwashita et al., 2003), but no direct link between neural pathology and cellular mechanisms in humans had been demonstrated. The use of iPSCs from three patients with FD, induced with lentiviral methods, demonstrated not only that the establishment of induced stem cells with the disease genotype was possible, but that iPSC lines differentiated to CNS lineages

showed lower levels of IKAP protein, reduced neurogenesis, and impaired migration of neural crest precursors (Lee et al., 2009). Previous work had demonstrated that lymphocytes from patients could be pharmacologically altered to increase IKAP protein levels (Slaugenhaupt et al., 2004), but the use of patient-derived iPSCs showed for the first time that the use of candidate drugs could be used to ameliorate the pathology in neural cells, specifically by treatment with kinetin, one of three compounds theoretically able to affect IKAP protein levels and its cellular functions (Lee et al., 2009).

Similarly, iPSCs have been used to investigate the degenerative processes involved in spinal muscular atrophy (SMA), a disorder of lower motor neurons known to result from mutations of the *smn* genes (Ebert et al., 2009). Electrically active human motor neurons have been generated from iPSCs (Karumbayaram et al., 2009) and with this technique, Ebert and colleagues demonstrated that these cells became smaller and reduced in number when derived from a SMA patient compared to those derived from a healthy family member. These differences essentially reproduced *in vitro* the features of *in vivo* disease, including spinal motor neuron protein aggregates. In a major advancement for drug discovery, iPSCs also allowed for the testing of valproic acid and tobramycin therapeutically, which reduced pathological protein aggregating in patient-specific cells and provided a litmus test for other possible therapeutics for SMA.

Amyotrophic lateral sclerosis (ALS) has also been studied using patient-derived iPSCs (Dimos et al., 2008). Unlike SMA and FD, ALS and many other neuropsychiatric disorders are not always the result of a mutation in a specific gene but likely have a range of different and even multiple origins in each particular patient. Dimos and colleagues derived iPSCs from an elderly patient with severe familial disease and were able to study the pathology of differentiated motor neurons in culture regardless of the advanced age or severe disease of the donor. While this patient had a known genetic mutation and the study of other patients with ALS may prove more challenging, the success of this first iPSC study on ALS holds great promise for determining even a few of the molecular mechanisms of this and other sporadic diseases.

Efforts using iPSCs to understand disorders affecting more rostral portions of the CNS are also underway, but, perhaps due to the complexity of brain cell structure and function, such investigations lag behind those examining the spinal cord and peripheral nerves. Yet, when ES cells and even iPSC-derived neuronal progenitors are cultured without growth factors and morphogens, they take on a forebrain fate and even show functional electrical activity (Hu et al.; Johnson et al., 2007; Pankratz et al., 2007). This appears to be a “generic” forebrain fate, conferred by the expression of OTX homeodomain genes and other transcription factors that determine brain fate early in development. The iPSC-derived cells need further patterning signals to generate specific subpopulations of forebrain neurons. How this forebrain fate occurs and what signals early neural cells and their progeny obey as they differentiate and become patterned into distinct subpopulations, are areas of active current investigation. Answering these questions will have a major impact on research into neuropsychiatric disorders which may alter the proportion of specific cell types and their fate. Published protocols suggest that mouse and even human ES cells can recapitulate the typical temporal patterns of development of cortical cells in culture, providing new models for studying this highly-coordinated sequence of events (Eiraku et al., 2008; Gaspard et al., 2008; Gaspard et al., 2009).

Many of the first advances on disorders of the rostral CNS have been made for those with known genetic mutations. Patient specific iPSCs are in the first stages of investigation for Down syndrome, Gaucher disease type 3, Huntington disease (Park et al., 2008) and epilepsy, with investigations of the latter particularly focusing on patients with known

sodium channel mutations such as *SCNA1*, the most commonly mutated gene in seizure disorders (Meisler et al., 2010). For Huntington disease, monkey iPSCs have already shown that differentiated neurons have disease-specific accumulation of huntingtin aggregates (Chan et al., 2010).

Work on iPSCs of other brain disorders with known genetic mutations has also demonstrated the need to be systematic in the evaluation of these reprogrammed cells, which may not consistently reproduce the development of typical stem cells. Skin and lung fibroblasts from three patients with Fragile X, a disorder characterized by intellectual disability and expanded triplet repeats in the fragile X mental retardation (*FMR1*) gene, were used to create iPSCs (Urbach et al., 2010). While Fragile X iPSCs met all basic criteria of stem cells, they differed from both wild type and Fragile X embryonic stem cells, in which the *fmr1* gene is active regardless of triplet repeats; the reprogramming process using lentiviruses was not able to reverse the methylation of *fmr1* that typically inactivates the gene as cells differentiate. However, iPSCs taken from Rett syndrome patients with MeCP2 gene mutations show that the reprogramming process can induce the normal active status of both X chromosomes in pluripotent cells (Marchetto et al., 2010). When cell lines were established from cells that had these “normal” reactivated pairs of X chromosomes, X chromosome silencing occurred with differentiation. Interestingly, Rett syndrome neurons showed abnormal synapse structure and function, a measurable phenotype for drug discovery trials, even in cells displaying normal expression of the MeCP2 gene from the active X chromosome, suggesting a non-cell autonomous mechanism of disease (Marchetto et al., 2010). These results showing varied gene silencing during reprogramming suggest that different techniques and/or different genotypes will have to be more thoroughly understood to fully utilize iPSCs, even in the case of disorders with known genetic mutations.

The most progress with iPSCs has been made on Parkinson disease (PD), an example of a disorder in which the cellular pathology—loss of dopaminergic neurons in the substantia nigra—is well understood, making it amenable to treatment with transplantation. iPSCs represent a potential source of transplantable cells that, if derived from the patient themselves, would be immunologically compatible. Dopamine neurons and cells expressing tyrosine hydroxylase have been derived from human and rodent iPSCs, albeit with low and variable levels of efficiency (Chambers et al., 2009). More recently, iPSCs have been generated from patients with PD (Soldner et al., 2009). Both undifferentiated and differentiated cells from these and non-patient derived iPSCs have been successfully transplanted into rodent models of PD showing the potential to correct behavioral and anatomical pathology (Cai et al., 2009; Hargus et al., 2010; Wernig et al., 2008). While iPSCs have not been demonstrated to be significantly better than embryonic stem cells in terms of this normalization, the early potential shown in these studies suggest that there may be equal promise for iPSCs in late-appearing neurodegenerative and early-appearing neurodevelopmental disorders.

Interestingly, the first disorder for which transplanted cells became an approved treatment option falls into a separate category entirely—spinal cord injury. Starting in 2009, the FDA granted approval to use human ES cells as an experimental treatment for spinal cord injury and, more recently, ALS. ES cells have a longer track record of success, with resolution of many of the risks yet to be resolved before iPSCs can be used in human disease treatment—namely the risk for tumor formation, graft rejection, DNA recombination in culture and appropriate telomerase levels to ensure cell survival (Naegele et al., 2010). Thus, several goals must be met before we may be able to apply the iPSC technology to tissue treatments for CNS disorders. However, as discussed above, iPSCs promise significant advances in cell-based regenerative medicine and other methods for improving treatments. Indeed, even

the early reports discussed here demonstrate how iPSCs have pinpointed disease mechanisms and tested successful candidate drugs for multiple disorders.

The most ambitious future application of iPSC technologies is to derive isogenic, relatively differentiated, transplantable cells to assist in the cure of neurodegenerative disorders. The hope is to provide cell replacement for neurological conditions such as Parkinson, Huntington and Alzheimer disorders. If the neurodegenerative conditions are driven by an intrinsic genetic defect, this will be likely reproduced in the transplanted isogenic cells, and therefore this strategy depends upon the ability to fix genetic disorders by *in vitro* gene replacement. However, there are concerns that will need to be addressed before iPSC and ES can be used for human neural cell replacement therapy, particularly the potential of these cells to accumulate point mutations and CNV/SVs during culture. Hence, their long-term genomic stability will need to be examined. Other concerns are potential tumor formation, arising from the intrinsic pluripotency of these cells or from the oncogenes that are used for their derivation. It is also significant that the differentiation of iPSCs *in vitro* will necessarily be different from that which occurs *in vivo*. Even proposals to use more naturalistic culture conditions may be limited by the complexity of the interactions that occur in the developing brain that involve neural, vascular, and immune cells and which are not fully understood. The neural cells derived from iPSCs may therefore differ from their *in vivo* counterparts in their stability and potential to continue or change development in response to extracellular cues. This may affect their potential as transplantable tissue.

Conclusions

The derivation of iPSC from specific individuals and their differentiation into neural cells provides the first opportunity to correlate individual genetic variation with patterns of gene expression and specific processes of neurodevelopment. We should be able to use this tool to begin to understand how specific genetic mutations affect the regulation of levels and function of mRNA transcripts in neurons and their progenitors, and study the impact of these changes for the differentiation of neural stem cells into neurons and glia. Immediate and long-term goals are to use iPSCs to understand aspects of the neurobiology of neuropsychiatric disorders and their genetics on the individual patient level, as well as develop novel diagnostic tools and pharmacological interventions.

These *in vitro* disease models do pose many challenges, but some can and have already been developed for disorders of both early development and later degeneration. Greater progress has been made for disorders with origins in early development, demonstrated by changes in early events such as reduced synaptogenesis in Rett syndrome. However, *in vitro* models of later degenerative illnesses may be just as informative, potentially revealing early, subtle changes in development that underlie later disease onset.

Any early or late phenotypes identified in differentiated cells derived from iPSCs may be used to test medications that could compensate for intrinsic pathology, as has been shown for SMA and familial dysautonomia. This approach may reveal new therapeutic uses for drugs that are already approved but may also allow for the large-scale testing of new compounds to correct whatever cellular process is defective, be it cell survival, cell adhesion, protein turnover, metabolism or synaptic process. It is unclear at this time whether the largely unknown processes leading to many disorders of the brain and spinal cord are reversible in this manner, but using *in vitro* disease models derived from iPSCs may be one of the first ways in which such questions can be asked.

Clinical research in developmental neuroscience may benefit from these individualized developmental models by discovering preventive measures for disease that were previously unthinkable.

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Figure 1.

Schematic outline of the goals achievable with the iPSC technology coupled to advanced cellular and genomic methodologies. In principle we should be able to connect genomic structure and mRNA transcripts expressed at different stages of neural development with the basic biological structure and function of neural cells, i.e., their number, size, neurochemistry, synaptic function, etc. The genomic structure can vary according to structural human polymorphisms or can be differentially expressed into transcripts under the influence of epigenetic events. These biological characteristics predict how cells will organize into circuits that eventually mediate cognitive functions and their dysfunction, i.e., neuropsychiatric disorders. The structure of the genome is thought to be mostly stable throughout development, whereas the transcriptome and epigenome vary widely at each epoch. The broken arrow reflects the recent discovery of retrotransposition events occurring specifically during neurodevelopment (Coufal et al., 2009; Muotri et al., 2010).

Abbreviation: SNPs, single nucleotide polymorphisms; CNVs, copy number variations; SVs, structural variations.

Figure 2.

By assembling human iPSCs into aggregates of about 9,000 cells and subjecting these aggregates to a step-wise differentiation protocol that converts cells into neural progenitors, we obtained neural structures with characteristics of anterior forebrain. At day 22 (A), cells are still undifferentiated; at day 50 (B-E) these structures, after sectioning and immunostaining, revealed layers of progenitors expressing Pax6 and nestin (B), surrounded by neurons expressing MAP2 (C-D). These neurons have forebrain character, as they do not express Zic1/2, a cerebellar marker, but express Ctip2 (D) and Tbr1 (E), transcription factors both expressed by developing neuron of the cerebral cortex.

Table 1

Derivation of iPSCs from human somatic cells.

Reprogramming Factors [*] /Cell type	Methods	Advantage	Disadvantage	References
O,S,K,M/Fibroblast	VSV-G pseudotyped retroviral particles	High transduction	Random genomic integration, low reprogramming efficiency	(Takahashi et al., 2007)
O,S,N,L/Fibroblast	VSV-G pseudotyped lentiviral particles	High transduction	Random genomic integration, low reprogramming efficiency	(Yu et al., 2007)
O,S,N,L ^{**} /Fibroblast	Transient plasmid DNA transfection using non-liposomal mixture of lipids	Simple and no need for viral packaging	Possibility of random genomic integration of DNA fragments, very low reprogramming efficiency	(Si-Tayeb et al., 2010)
O, S, K, M/Fibroblast	Electroporation of single multiprotein expression vector	Simple and no need for viral packaging, high reprogramming efficiency	Possibility of random genomic integration of DNA fragments	(Kaji et al., 2009)
O, S, K, M, N, L, SV40/Fibroblast	Electroporation of non-integrating episomal vectors	Simple and no need for viral packaging	Possibility of random genomic integration of DNA fragments, low reprogramming efficiency	(Yu et al., 2009)
O, S, K, M/Fibroblast	Inducible single polycistronic lentiviral vector	Cre-mediated excision of the single reprogramming cassette	Random genomic integration, after Cre-mediated excision leaving LTR with a single loxP site, very low reprogramming efficiency	(Carey et al., 2009; Soldner et al., 2009)
O, S, K, M/Fibroblast	Piggyback transposon/transposase system	Complete removal of single expression cassette of reprogramming factors, high reprogramming efficiency	Inefficient excision, laborious and another transient transfection step needed	(Woltjen et al., 2009)
O,S,K,M/Fibroblast	Adenoviral vectors	Free of any viral or transgene integration	Laborious, requiring virology knowledge, strict biosafety regulations, low	(Zhou and Freed, 2009)

Reprogramming Factors [*] /Cell type	Methods	Advantage	Disadvantage	References
			reprogramming efficiency	
O, S, K, M/Fibroblast	Sendai virus vectors	Free of any viral or transgene integration, high reprogramming efficiency	Laborious, requiring virology knowledge, strict biosafety regulations	(Fusaki et al., 2009)
O, S, K, M/T cells	Sendai virus vectors	Free of any viral or transgene integration, high reprogramming efficiency	Laborious, requiring virology knowledge, strict biosafety regulations	(Seki et al., 2010)
O, S, K, M/Fibroblast	Protein transduction	No genetic modification	Very low reprogramming efficiency	(Kim et al., 2009)
O, S, K, M/Fibroblast	In vitro transcribed modified mRNA	No integration, very high reprogramming efficiency	Daily transfection of modified mRNA cocktail for 16 days	(Warren et al., 2010)

* O, Oct4; S, Sox2; K, Klf4; M, c-Myc; N, Nanog; L, Lin28; SV40, SV40 large T antigen

** Plasmid DNAs of the same lentiviral vectors of Yu, et al., 2007