Probing Disorders of the Nervous System Using Reprogramming Approaches

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Introduction

Diseases of the nervous system represent an enormous burden for society in terms of human suffering and financial cost. While significant advancements have been achieved over the last few decades particularly in terms of genetic linkage, clinical classification, and patient care, effective treatments are lacking. The inaccessibility of the relevant tissues and cell types in the CNS and the complex, multifactorial nature of most neurological disorders have hampered research progress. Animal models have been crucial in the investigation of disease mechanisms, but fundamental developmental, biochemical, and physiological differences exist between animals and humans. The importance of utilizing human cells for these purposes is evident by the large number of drugs that show efficacy and safety in rodent models of diseases but subsequently fail in human clinical trials, failures that are attributed partly to these species differences (Rubin, 2008). Furthermore, the overwhelming majority of neurological disease is of a sporadic nature, rendering animal modeling ineffective, while it remains unclear whether the relatively rare monogenic forms of disease truly represent the vast majority of sporadic cases.

The simultaneous development of methods for reprogramming adult cells into induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008) and the directed differentiation of pluripotent stem cells into distinct neuronal subtypes (Williams et al., 2012) suggested an attractive route to a novel model system for the study of neurological disorders. Patient-specific iPSCs can be generated using epigenetic reprogramming of various adult cell types, such as skin fibroblasts and blood mononuclear cells, and just like embryonic stem cells (ESCs), self-renew indefinitely and retain the potential to give rise to all cell types in the human body (Takahashi et al., 2007). More recently, sophisticated lineage-conversion approaches have allowed for the direct generation of neurons and neural cell types from adult cells by means of overexpressing key transcription factors (Tsunemoto et al., 2014). These methods have overcome some of the limitations of directed differentiation and have enabled the generation of cell types that, in many cases, were previously unattainable.

The overwhelming advantages of using iPSCs and lineage conversion to develop models of diseases of the nervous system are that they allow one to study disease mechanisms in the context of human neurons and in the context of each patient's unique genetic constellation. In many cases, established differentiation protocols allow for the generation of the particular neuronal subtype that is most vulnerable to the particular disease, such as spinal motor neurons (Davis-Dusenbery et al., 2014) and dopaminergic neurons (Kriks et al., 2011). These neurons can be produced in abundance from variable genetic backgrounds and could provide useful platforms for drug discovery.

The concept of using iPSCs and lineage conversion to study neurological disease appears straightforward: both approaches allow for the generation of patient-specific neurons, which are relevant to the disease of interest. In addition, when these neurons are compared with neurons generated from healthy controls, any differences identified could be related to the disease. In practice, however, this approach has proven to be more challenging than initially believed. What is the right cell type to make and study? How should quality control of neurons be performed? What are the right controls to use when assessing a disease-related phenotype? How do phenotypes identified *in vitro* relate to the clinical presentation of patients? These are just some of the questions that the community has struggled with since the initial description of iPSCs and the onset of the development of *in vitro* patient-specific disease models. Perhaps the seemingly biggest advantage of this approach—the ability to study disease in the genetic background of the patient—has created the biggest challenge, as genetic background contributes to high variability in the properties of the patient-derived cells. This variability is a reality that neurologists have been facing for years, as often, two patients diagnosed with the same condition might present with very different clinical profiles. The technology of cellular reprogramming has brought this reality of clinical heterogeneity seen in patients from the bedside to the lab bench.

Since the initial description of reprogramming technologies, neuroscientists, neurologists, and stem cell researchers have generated and characterized hundreds of patient-specific stem cell lines as well as neuronal cells derived from them. The first "wave" of disease-modeling studies focused on generating patient-specific human neurons and confirming previously described pathologies (Dimos et al., 2008; Ebert et al., 2009; Marchetto et al., 2010; Brennand et al., 2011; Seibler et al., 2011; Bilican et al., 2012; Israel et al., 2012). More recent studies have revealed novel insights into disease mechanisms and employed gene editing approaches to clearly demonstrate the association of identified phenotypes with known genetic variants that contribute to disease (An

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et al., 2012; Corti et al., 2012; Fong et al., 2013; Reinhardt et al., 2013; Kiskinis et al., 2014; Wainger et al., 2014; Wen et al., 2014). At the same time, our ability to generate neuronal subtypes via directed differentiation and the exogenous expression of transcription factors has made tremendous progress.

Specificity of Phenotypes: The Importance of Controls

Significant technical advancements achieved during the past few years have allowed for the generation of patient-specific iPSCs that are free from genomic integration of the reprogramming factors (Malik and Rao, 2013). The essential quality of any newly derived iPSC can be easily assessed by (1) immunocytochemistry for pluripotency markers (e.g., NANOG/SSEA3); (2) a quantitative pluripotency assay, such as TaqMan® hPSC Scorecard™ Assay (ThermoFisher Scientific, Waltham, MA) or PluriTest™ (Scripps Research Institute, available at pluritest.org); and (3) analysis of genomic integrity (e.g., karyotyping, array comparative genomic hybridization).

Disease-modeling studies based on iPSC technology have relied on the use of diseased cells derived from patients as a model for disease and cells derived from healthy individuals as controls. However, genetic and potentially epigenetic heterogeneity of iPSC lines contributes to functional variability of differentiated somatic cells, confounding the evaluation of disease-modeling experiments (Sandoe and Eggan, 2013). Such variability can be introduced at multiple levels, including the generation of stem cell lines, continuous *in vitro* culture, variation in cell culture reagents, differential efficiencies of neural generation, and genetic background. Different approaches can be taken to overcoming this variation. One approach is through the use of targeted gene editing that results in the generation of a control stem cell line that is isogenic to the patient one, except for the disease-causing mutation. Such an approach effectively minimizes line-to-line differences and is a crucial tool for iPSC-based disease modeling.

CRISPR/Cas9, a recent technology that has emerged, allows for the efficient generation of such isogenic stem cell lines (CRISPR stands for clustered regularly interspaced short palindromic repeats, and Cas9 is a class of RNA-guided endonucleases) (Hsu et al., 2014). The system contains two essential components: an enzyme that can cleave DNA so that a double-strand break or a single nick is generated, and a guide RNA that targets the enzyme to a specific genomic location. By simultaneously introducing either a single-stranded oligodeoxynucleotide containing the desired edit, or a targeting plasmid with larger desired sequence alterations, the genomic sequence can be precisely edited via the cells' own endogenous repair mechanism, homologous recombination. Given the incredible versatility of the CRISPR/Cas9 system and the continuous evolvement of the technical aspects of this approach, it should be expected that every iPSC study that focuses on genetic forms of disease should include an isogenic control cell line. The rescue of a phenotype by genetic correction can lead to the conclusion that the genetic lesion is necessary for the onset of the phenotype. The same technique can be used to introduce a disease-associated mutation in a healthy iPSC line in order to assess whether the mutation in itself is sufficient for the onset of particular phenotypes.

An alternative approach to the concern of variation would be to utilize multiple stem cell clones from each individual patient and compare the desired measurement against multiple healthy individuals. The use of multiple patient clones would ensure that the phenotype is not an artifact of a defective clonal cell line, while the use of multiple healthy controls should encapsulate sufficient technical and genetic variation so that the measured cellular properties (e.g., neuronal firing, dendritic density) will represent a true average. This approach will be important in studies of sporadic disease.

An important point to consider when assessing the specificity of an identified phenotype is whether it is apparent only in the cell type known to be most vulnerable to the disease being modeled. In amyotrophic lateral sclerosis (ALS) patients, for example, it is the upper and lower motor neurons that are initially targeted by disease mechanisms and gradually lost, while sensory neurons remain relatively unaffected. It would therefore be predicted that a phenotype that is truly relevant to the disease would not be evident in a sensory neuron generated from the same individual. Although this approach could be valuable, it should be taken with caution for two reasons: (1) because a sensory neuron might simply be resistant to a phenotype, and therefore it is the effect of the phenotype on the sensory cell that should be considered, not simply the presence of the phenotype in itself; and (2) because it might be the *in vivo* microenvironment of a sensory neuron that confers resistance and not a cell-autonomous trait. Nevertheless, studies have demonstrated neuronal-type specificity of certain phenotypes. These include the sensitivity of mutant Parkinson's disease (PD) tyrosine hydroxylase (TH)–positive neurons but

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not TH-negative neurons to H2O2-induced toxicity (Nguyen et al., 2011) and morphometric deficiencies of mutant ALS, Islet (ISL)–positive motor neurons but not ISL-negative neurons grown in the same culture dishes (Kiskinis et al., 2014).

A major advantage of using reprogramming approaches to study neurological disease is the ability to assess the biological variation associated with a specific neuronal defect. Consider that a phenotype (e.g., defective lysosomal function) has been identified in neurons derived from a patient cell line and that this phenotype is mutation dependent (i.e., it is corrected in an isogenic control line). The first level of biological variation can be addressed by examining neurons derived from a different individual that harbors the exact same mutation in the same gene. If the phenotype is not present, then additional genetic or epigenetic factors might be necessary for the onset of the defect. The next level of biological variability can be addressed by examining neurons from a patient with a different mutation in the same gene. Lastly, the broader relevance of the identified phenotype for the disease can be assessed by examining the lysosomal function of neurons from patients with mutations in different disease-causing genes as well as in a large number of sporadic cases.

A Shift in Focus: From Developing Neurons to Maturing and Aging Them

A critical area that deserves further investigation is the maturity and aging of cells derived *in vitro*. We like to think that there are three stages we need to consider when setting up *in vitro* models of disease: the development, the maturation, and the natural aging process of a neural cell type. Although significant advancements have been achieved in generating and maturing neural cell types (either by directed differentiation or lineage conversion), little has been done in terms of affecting the aging of cells. For late-onset diseases such as ALS, frontotemporal dementia, Huntington's disease (HD), PD, and Alzheimer's disease (AD), it is possible that changes elicited by aging are required to induce the disease process. Age is the strongest risk factor for neurodegenerative diseases, and although there are rare cases with early-onset presentation, the overwhelming majority of patients develop clinical symptoms in the later stages of their lives. The nature of age-related risk remains largely unknown, and whether it arises from cell-autonomous mechanisms or as a result of a systemic dysfunction remains to be determined. A number of studies support the notion that cellular epigenetic changes in the CNS correlate with aging. For example, recent work has demonstrated that profound changes in DNA methylation levels occur in the brains of mice with age (Lister et al., 2013), while aging oligodendrocytes lose their ability to effectively remyelinate damaged nerves (Ruckh et al., 2012). Importantly, under conditions of heterochronic parabiosis in mice, the effects on oligodendrocytes were reversible, implicating some aspect of epigenetic regulation.

Current studies suggest that the transcriptional and electrophysiological properties of both iPSC-derived and lineage-converted neurons are more similar to fetal neurons than adult ones (Son et al., 2011; Takazawa et al., 2012). It is likely that extrinsic factors present during normal development or aging are required to activate the maturation process. We and others have shown, for example, that adding primary astrocytes to lineage-conversion cultures significantly improves the maturation of induced neurons (Son et al., 2011; Chanda et al., 2013; Wainger et al., 2015). Additional progress in generating more mature and aged cells will require a better understanding of the gene expression and functional changes associated with maturation and aging. This has been difficult to obtain for specific neuronal subtypes because of the scarcity of available human tissue. Efforts such as those of the Allen Brain Institute have shed some light on these markers, but future studies will need to analyze specific neuronal subtypes in order to be sure that differences between aged neurons and young neurons are truly the result of aging and not of different neuronal subtypes.

In addition to glial-derived factors, Rubin and colleagues recently showed that circulatory factors contribute to the aging process in the CNS (Katsimpardi et al., 2014). They were able to identify a single factor: growth differentiation factor 11 (GDF11), whose expression normally declines with age. Interestingly, restoring GDF11 levels in old mice rejuvenated the proliferative and neurogenic properties of neural stem cells in the mouse (Katsimpardi et al., 2014). This finding suggests that other factors may control the aging of neurons and could be exploited to regulate this process *in vitro*.

Studer and colleagues took a more intrinsic approach to inducing aging in iPSC-derived neurons by expressing progerin, which is a mutant form of the Lamin A protein that causes accelerated aging phenotypes in humans (Miller et al., 2013). The expression of progerin induced higher levels of DNA damage and mitochondrial reactive oxygen species

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in dopaminergic neurons derived from PD patients, which enabled the detection of PD-associated disease phenotypes such as dendrite degeneration, mitochondrial enlargement, Lewy body–precursor inclusions, and suppression of TH expression (Miller et al., 2013). It remains unclear whether this approach induces the recapitulation of *bona fide* disease processes, but it does represent a new line of targeted aging procedures.

From Cell Autonomy to More Sophisticated Systems

Neurons do not exist in isolation in the human nervous system. Rather, they form elaborate and functional networks with other neurons and rely on a sophisticated microenvironment that is created by the interactions with other neural and nonneural cell types, which provide structural, metabolic, and functional support as well as effective communication (Abbott et al., 2006). Glial cells, astrocytes, oligodendrocytes, microglia, and endothelial cells exist in abundance in the nervous system and play vital functional roles. Glial cells buffer harmful ions, astrocytes provide nutrients and circulate neurotransmitters around synapses, oligodendrocytes form myelin sheaths around axons, microglia scavenge and degrade dead cells, and endothelial cells are important for maintaining the blood–brain barrier. Cell–cell interactions and the microenvironment as a whole might mediate important neuroprotective or neurotoxic activities in response to disease or injury. In fact, a number of studies during the past few years have clearly demonstrated that non–cell-autonomous processes involving astrocytes, oligodendrocytes, and microglia play a critical role in mediating disease progression and, potentially, onset in neurodegeneration in such diseases as ALS, HD, PD, prion disease, the spinal cerebellar ataxias, and AD *in vivo* (Ilieva et al., 2009). The strength of utilizing iPSCs to study neurological disease is found in their ability to generate a range of different cell types from the same genetic background. This versatility allows for the assessment of how, for example, a specific genetic lesion might differentially impact neuronal subtypes. It also allows for a rational step-by-step approach for assessing how cellular interactions might contribute to the evolution of a disease-associated phenotype or a cellular response to stress.

The coculture of spinal motor neurons with cortical astrocytes has been utilized in one of the first stem cell–based models of ALS to demonstrate how mutant or healthy astrocytes significantly compromise or maintain, respectively, the health of a pure population of motor neurons (Di Giorgio et al., 2008; Marchetto et al., 2008). The coculture of cortical excitatory with cortical inhibitory neurons, and the establishment of functional circuitry, might be beneficial when studying epileptic syndromes. The clinical presentation of epileptic patients is the result of the functional control (or lack thereof) of a network of neurons, so recapitulating such a network could be an essential step toward the development of a cellular disease model. The importance of the local microenvironment to neuronal function (and potentially, dysfunction during disease) is also relevant in the context of the three-dimensionality that it creates. Neither the brain nor the spinal cord hosts isolated neurons surrounded by an entirely liquid trophic support (akin to culture media) in which nutrients, molecules, and proteins can freely diffuse and float around. Recently, Kim, Tanzi, and colleagues were able to successfully recapitulate amyloid-beta (Aβ) plaques and tau neurofibrillary tangles—the two pathological hallmarks of AD—in a single three-dimensional human neural-cell culture system (Choi et al., 2014). Although this system was not based on iPSCs, and their cell lines expressed slightly elevated protein levels of *PSEN1* and *APP*, they designed a simple but innovative cell culture system with neurons grown embedded within a 0.3 mm layer of an extracellular matrix composed of BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Erembodegem, Belgium). This viscous layer reduced the diffusion of secreted Aβ and led to the accumulation of aggregated plaques. This was the first time this had been achieved in a cell-based *in vitro* system and demonstrates the importance of using a three-dimensional environment for disease-modeling assays.

The recent description of cerebral organoids generated from human pluripotent stem cells and resembling the three-dimensional regional organization of a developing brain has created an exciting opportunity for iPSC-based disease-modeling approaches (Lancaster et al., 2013). These brain-like structures, formed by the combination of external growth factor patterning and intrinsic and environmental cues, exhibit distinct regional identities that functionally interact and, most importantly, recapitulate human cortical organization. The authors utilized this method to study microcephaly and demonstrate that patient-specific organoids show premature neuronal differentiation and are capable of developing only to a smaller size. Significantly, mouse models have failed to effectively recapitulate these disease

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phenotypes for microcephaly, probably owing to the dramatic differences in the development and regional organization of their brain, as mice do not have an outer subventricular zone. This system may be suitable for the study of other neurodevelopmental and neuropsychiatric syndromes in which moderate but crucial defects in cortical organization and function are present. This approach also may be useful for recapitulating human neurodegenerative models that primarily affect brain function because it may allow for the establishment of neuronal circuitry as well as biochemical networks.

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