11

Using a Stem Cell-Based Approach to Model Neurodegenerative Disease

Where Do We Stand?

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Abstract

Interest in improving methods to study human disease is rapidly growing, with the goal of providing effective therapeutics for serious, complex disorders. Following the discovery of cellular reprogramming, much of this interest has focused on the role of induced pluripotent stem cells (iPSCs). The notion is to use patient-derived iPSCs to produce differentiated cells with disease genotypes and phenotypes. These differentiated cells, theoretically available in unlimited numbers, can then be used to analyze disease pathology as well as to identify and test therapeutics in a highly personalized manner. The idea is simple, but execution difficult. This chapter reviews the advantages and complexities of this new approach.

Introduction

For over a decade, the limitations of model systems used to study human disease have been discussed and dissected. Driving this, in part, was the recognition that mice are only capable of representing certain components of human physiology, as can readily be observed in the context of neurodegenerative disease. For example, the standard mouse model used to study amyotrophic lateral sclerosis (ALS) involves significant overexpression of mutated *SOD1* and is problematic for several reasons: *SOD1* mutations account for just a small percentage of ALS cases. In addition, the accelerated course of the mouse version of the disease, which is presumably due partly to *SOD1* overexpression, makes the mouse easier to study, but it does not accurately reproduce the

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human condition. For Alzheimer disease (AD), the "triple transgenic" mouse engineered to express three separate disease-related mutations seems advantageous because it reproduces, in a reasonable timeframe, much of the pathology and some of the behavioral defects found in AD patients. However, it is again artificial; none of the mutations expressed in the mouse model have been found in individual patients, leaving some investigators to question whether studying this mouse can provide any disease-relevant information.

An even better example may be the set of psychiatric diseases that is presumably more heterogeneous and complicated than either ALS or AD. In numerous, provocative papers, Hyman discussed the "mouse problem," stating in no uncertain terms that neither new information nor new therapeutics have arisen in decades, owing in part to the use of mouse models and their inability to represent complicated behavioral human diseases (Hyman 2014). Differences in cortical development between mouse and humans may be to blame, but the problem is not confined to just neurological and psychiatric disorders. In a recent article, Seok et al. (2013) described how inadequately mouse models of sepsis mimic the human clinical condition.

Of course, as described by Robbins (this volume), mouse models may be used to make increasingly reliable predictions concerning human pathophysiology. However, logically, human disease is best studied using diseased cells from humans. Historically, for neurodegenerative diseases, this has only been possible through postmortem tissue. The disadvantages from this are obvious. Conclusions drawn from such analyses resemble a CSI (crime scenes investigation) type of method: scientists try to surmise what happened during the course of the disease to produce the changes observed in postmortem tissue. The focus is on pathology, rather than on physiology. However, notably, postmortem tissue often lacks the very cells that were affected by the disease, since they have already died. In spinal cord biopsies obtained postmortem from ALS patients, for example, very few motor neurons remain. In AD, neurological scientists are still debating whether amyloid plaques, a pathological hallmark of the disease readily seen in AD patient brains, are anything more than tombstones, denoting that something bad has happened, but not proving that plaque formation causes the disease.

Everyone agrees that we need to be able to study the progression of disease at its earliest stages, and introduce interventions accordingly, but how can this be done? Early intervention is particularly important as recent observations suggest that underlying disease-related changes in familial cases of AD occur over a 20-year period, and this could be characteristic of other degenerative diseases. Tremendous advances in imaging and biomarker analysis are beginning to reveal some of these earlier changes. In addition, genome sequencing is pointing investigators toward disease-related genes, and that type of information has already been extremely useful to cancer biologists. Nonetheless, having *in vitro* human cell-based systems that are capable of recreating diseases

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from beginning to end would likely contribute substantially to our knowledge of neurodegenerative diseases.

Problems that scientists experience in studying disease become magnified when the research shifts to identifying therapeutics that work. Again, it is reasonably obvious that an imperfect understanding of human disease progression will lead to suboptimal targets for drug discovery. In addition, the limited availability of faithful mouse models, combined with access to postmortem human tissue alone, means that many therapeutics are focused on late, rather than early, events. The inherent disadvantages associated with studying cells already dying are exacerbated by other flaws in the drug discovery system practiced virtually universally in the pharmaceutical industry. The problem can be defined in quite a simple way: too much money (billions of dollars per drug) is spent developing far too few breakthrough treatments. Even worse, more and more money is being spent, more and more screens are being done, but the number of new drugs is not increasing concomitantly. The efficiency of the entire process is shockingly low, in addition to being (perhaps) unavoidably lengthy.

Numerous articles attempting to understand the underlying causes of these issues have been published, and many possibilities have been raised:

- All of the easy-to-discover drugs have already been found.
- Individual target-based approaches are less good than strategies aimed at correcting overall disease phenotype.
- The nature of the chemical matter used for drug screens and drug development is suboptimal.
- Decision making is poor.

Here I focus on a simple, not yet validated, hypothesis: namely, the process of finding safe and effective drugs will improve greatly if drug discovery campaigns place human cells—diseased and control—front and center. This hypothesis is based on the fact that most pharmaceutical projects use highly engineered cells or cells which are not disease relevant (e.g., motor neuron diseases have never been studied in human motor neurons) in screening and *in vitro* efficacy testing. Furthermore, drug safety is established (by FDA requirement) in two nonhuman species so that human safety data are not available preclinically. Finally, there is no current method, at least for the bulk of neurodegenerative diseases, to match specific treatments to individual patients who may most benefit from them. Most neurodegenerative diseases are heterogeneous, reflecting individual mutations (often in an extremely small percentage of patients), environmental components, and/or aging. Thus, it is perfectly possible that at least a subset of patients with particular neurodegenerative diseases could have been treated with specific drugs that are ineffective on the majority of patients enrolled in a trial (Engle and Puppala 2013; Merkle and Eggan 2013; Yu et al. 2013a).

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History of the iPSC Approach to the Study and Treatment of Human Disease

In 2002, Jessell and colleagues described a simple protocol to produce spinal cord motor neurons from wild-type mouse embryonic stem (ES) cells using just two small molecules: retinoic acid and an agonist of hedgehog signaling (Wichterle et al. 2002). This method was a successful attempt to approximate embryonic spinal cord development. A surprisingly small number of investigators recognized this discovery as a key enabler of a new approach to studying disease. In essence, this paper showed that it was possible, for the first time, to produce large numbers of motor neurons—billions of them—when, previously, scientists had access to thousands or, in the extreme, hundreds of thousands of those cells. Furthermore, this method could be applied to mouse ES cells expressing various mutant genes, such as those found in ALS and the genetic motor neuron disease spinal muscular atrophy (SMA). In other words, investigators could viably produce control and diseased mouse motor neurons in large enough numbers to carry out drug screens. A few years later, it was shown that motor neurons could also be produced from human ES cells using a similar protocol (Amoroso et al. 2013).

One reason that this concept failed to catch on was that it relied on the use of ES cells. This was particularly controversial in the pharmaceutical world, which tends to be inherently conservative. Another limitation revolved around poor availability of human ES cells, especially those carrying disease genes. Both issues disappeared with the discovery of cellular reprogramming, the generation of induced pluripotent stem cells (iPSCs) from skin and blood, by Yamanaka and colleagues (e.g., Takahashi et al. 2007; Takahashi and Yamanaka 2006; Yamanaka 2007). It became possible—indeed, even practical—to produce iPSCs from tens or hundreds of patients with diseases of interest. It also suggested a conceptually simple way of establishing assay systems focused squarely on differentiated human patient cells recapitulating human disease processes (Rubin 2008). Fully implemented, this approach can be broken down into a series of seemingly straightforward steps:

1. Identify appropriate numbers of patients with disease of interest and obtain skin or blood samples.
2. Produce clones of iPSCs (typically three well-characterized clones) from each patient.
3. Differentiate iPSCs into neuronal type(s) affected by the disease of interest (e.g., motor neurons for ALS and SMA).
4. Establish culture conditions so that relevant phenotypes can be observed.
5. Follow the progression of the "disease in a dish" so that its earliest deviations can be pinpointed.

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6. Validate findings from steps 4 and 5 across an array of individual patients to ensure that they are observed in more than a single set of patient cells.
7. Use this information to discover appropriately screenable targets (e.g., receptors, kinases) or phenotypes (e.g., decreased synapse number).
8. Screen appropriate libraries (small molecules, biologics, siRNAs) against the target or phenotype using cultures of diseased neurons.
9. Test best hits (before and after chemical optimization, when appropriate) on neurons produced from multiple patients. This information becomes part of the preclinical *efficacy* package.
10. Test efficacious compounds for safety on individual patient-derived cardiac myocytes and hepatocytes. These results constitute part of the preclinical *safety* package.
11. Test best compounds on groups of patient neurons prior to a Phase I study. Choose most responsive patients for enrollment.

In short, if this concept were to be validated (ultimately in the clinic), the outcomes would be significant. It could lead to an improved understanding of various types of neurodegenerative disease and result in better targets for drug identification. More relevant cells would be available for screening and safer, more efficacious therapeutics could be developed. Our ability to test particular drug candidates on individual patient cells would be enhanced and clinical trials initiated using specific patients who are most likely to benefit from candidate drugs.

Implementation of the Method

In this section, I highlight various considerations related to particular steps that will be required to use iPSCs for drug discovery and other types of studies.

Production of iPSCs

Unfortunately, none of the steps involved in this method is without controversy. Many papers have addressed issues related to cellular reprogramming, some of which are worth emphasizing (Cahan and Daley 2013; Liang and Zhang 2013). The first is that iPSCs are generally clonal: each person is, in essence, represented by the progeny of one cell, and the cell-to-cell variability in gene expression and in mutational burden is well known.

Currently, multiple methods are employed to produce iPSCs. While they may be functionally similar, the reprogramming achieved by all of them is thought to be both incomplete (with cells retaining an epigenetic memory of their tissue of origin) and capable of introducing additional genetic and epigenetic variability. Furthermore, while it has been clear for several years that

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human ES cell lines differ, iPSCs, although ES-like, are even more variable. Hence, there is an understandable lack of clarity surrounding the question of how many iPSC lines should be used for each individual patient. Similarly, because there is patient-to-patient variability among individuals even with simple monogenic diseases, it is not entirely clear how many patients should be used to represent a disease cohort. This problem is magnified enormously when studying heterogeneous diseases, such as psychiatric disorders.

Differentiation of iPSCs into Neurons

Surprisingly, motor neuron production from human ES cells or iPSCs is still one of the most efficient differentiation systems available. To produce other neuronal types (including cortical neurons, excitatory and inhibitory and midbrain dopaminergic neurons), production techniques are available. Three features characterize most of the differentiation methods, which, as with motor neurons, are generally based on recapitulating mouse embryonic development. First, most are extremely time consuming: producing neurons from stem cells can easily take one to four months, in addition to the three-month period required for iPSC generation. Second, there is surprising variability in the efficiency of the different methods, even when practiced by scientists within individual labs. Third, neurons in the cultures may be too immature to model late onset diseases accurately (discussed further below).

The variability issue is widely recognized. Most cultures described in the literature contain 20% or so of the desired cell product, in addition to residual progenitors, other types of neurons and, eventually, glial cells. Some neurobiologists assert that mixed cultures are preferable since this may reproduce the *in vivo* environment more accurately. However, the variability and heterogeneity of the cultures present challenges for some types of investigation. For example, microarray and RNA sequencing studies that compare diseased and control neural cultures may be compromised by the variability of cellular composition in the cultures. This is also true for some kinds of phenotypic assays (e.g., synapse formation) where results can be affected simply by having variability in percentages of the component cell types. Although it appears that particularly effective cocktails of inducing molecules can partially overcome intrinsic differences among iPSC lines, it may be overly optimistic to imagine that standard differentiation protocols will ever become efficient enough to produce completely uniform populations of individual kinds of neurons. Thus, it will be important to have the ability to purify desired neuronal subtypes. In the near future, this will be possible by using new genome-editing techniques (TALEN, CRISPR) to introduce cell type-specific reporters or by identifying specific sets of surface markers that allow fluorescence-activated cell sorting purification of neuronal populations. Purification of different types of neurons can be followed by remixing them, so that defined cultures with multiple

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neuronal types can be prepared. While technically feasible, this method may still not capture all of the biology of live brain tissues.

Other Ways to Derive Neurons from Patient Biopsies

Recently, differentiated cells have been obtained from patient fibroblasts using techniques that do not require an iPSC intermediate. These methods are generally referred to as transdifferentiation, direct differentiation, or lineage reprogramming and use the underlying principle of reprogramming (i.e., the dominant action of particular, often small, sets of transcription factors). For instance, expressing high levels of small numbers of transcription factors found in embryonic neurons and their progenitors is sufficient to convert fibroblasts to neurons (Chanda et al. 2013). Reassuringly, induced neurons made from mice expressing neuroligin-3 mutations displayed a synaptic defect similar to those seen in mouse neurons *in situ*. Other interconversions are also possible; for example, astrocytes overexpressing neurogenin-2 become neurons *in vitro* and *in vivo* (Guo et al. 2014). Surprisingly, this technique does not produce more uniform cultures than more standard differentiation methods. However, since it bypasses the reprogramming step, it can produce differentiated cells in weeks, rather than months. One possible disadvantage is that there is no recognizable neural progenitor stage between fibroblasts and neurons, since differentiation does not occur through a normal developmental sequence. Thus, if an investigator desires to produce large numbers of neurons, the starting fibroblasts themselves have to be expanded. At some point, highly expanded fibroblasts, especially those obtained from older patients, may senesce or experience a reduced ability to differentiate. A similar and promising concept has been applied to iPSCs which, following expression of the single transcription factor neurogenin-2 and puromycin-based selection, can produce electrophysiologically active cortical-type neurons in relatively good purity (Zhang et al. 2013).

Functional Validation of Neuronal Identity

What are the best ways to characterize stem cell or fibroblast-derived neurons? On the surface, this may seem like a trivial question. For example, motor neurons differentiated from stem cells express the motor neuron transcription factor Hb9, synthesize acetylcholine, innervate skeletal muscle cells in culture, and send axons to limb muscle when transplanted into chick embryos. However, these motor neurons have a rostral-type identity and must be treated with additional factors to form more caudal motor neurons or neurons found in particular motor pools.

Other types of neurons have been evaluated in similar ways. Which transcription factors and other individual markers (e.g., transporters, neurotransmitter receptors, synthetic enzymes) do they express? Do they have normal membrane potentials, produce synaptic proteins, and form spontaneously

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active synapses? Often investigators will call neurons "mature" if they express a small number of appropriate markers, fire action potentials, and form synapses. However, these cells are not truly mature since embryonic and fetal neurons express the same markers and also form synapses.

A stricter criterion, one preferred by more rigorous neurobiologists, judges cell functionality after transplantation. For example, Sudhof and colleagues found that induced human neurons were physiologically active when analyzed six weeks after transplantation into newborn mouse brain and were even capable of receiving synaptic input from existing mouse neurons (Zhang et al. 2013). Similarly, Espuny-Camacho et al. (2013) transplanted immature human cortical neurons into neonatal mouse brain and found that after several months they projected extensively and were able to form synapses with the host neurons. A higher-order demonstration of functionality would be similar to one achieved by Macklis and colleagues (Czupryn et al. 2011), who transplanted immature neurons *purified from normal embryonic hypothalamus* into brains of postnatal leptin-deficient mice and achieved behavioral rescue.

Discussions of how to define cell identity are not without basis. For example, recent work indicates that cells derived directly from fibroblasts maintain (by gene expression) a partial fibroblast character even when they show properties of other types of differentiated cells. Interestingly, it appears that cells can be functional, to at least some degree, even with a mixed identity. Perhaps of more concern are other sets of new experiments which seem to demonstrate that all *in vitro* differentiation protocols, which may closely mimic but are not identical to the *in vivo* environment, can only generate cells that are similar (but again not identical) to their "real" counterparts. This may mean that a compromise definition for cellular identify may need to be adopted—one based on functional outcomes, such as recapitulating properties of particular diseases.

How Mature Are Neurons That Are Differentiated from iPSCs (or Fibroblasts)?

From a purist's point of view, transplantation experiments measure only the potential of cells to function *once delivered to an appropriate* in vivo *environment* (i.e., they may not have been truly functional prior to transplantation). This is an important distinction because a variety of studies now suggest that most cell types produced from stem cells are quite immature. Given that *in vitro* differentiation methods are designed essentially as near (or not-so-near) copies of *in vivo* embryonic processes, it is not surprising that the cells produced are immature. However, the fact that most types of cells do not appear to mature appreciably under standard culture conditions is of more concern. Famously, pancreatic β-cells of stem cell origin are poor responders to high glucose; they only develop this capacity after weeks of being injected into a mouse kidney capsule. For researchers interested in modeling late onset neurodegenerative

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disorders, this is particularly significant. While it may be that immature neurons are still an advance over nonneuronal cells, a strategy for producing more mature cells is needed. Intriguingly, Sasai (2013) and Lancaster et al. (2013) utilized three-dimensional suspension cultures and found that, under those conditions, stem cells display a surprising amount of self-organization, constructing organ-like tissues of different kinds. Still, even the most organ-like individual tissue is still an individual tissue and will not reproduce cell phenotypes that rely upon more complicated interactions among organs.

Using Patient Neurons to Study Neurodegenerative Diseases

What Types of Diseases Can Be Studied?

From the perspective of disease modeling, nervous system disorders can be distributed into different categories: (a) primarily familial versus primarily idiopathic, (b) monogenic versus polygenic for familial forms of disease, (c) early onset versus late onset, and (d) cell autonomous versus noncell autonomous. Most investigators agree that early onset monogenic cell autonomous diseases are the easiest to study, whereas late onset, predominantly idiopathic, diseases are the hardest. Practically speaking, most neurological disorders involve multiple cell types, often including glial cells and immune cells, to some degree. However, many disease models are only expected to reproduce the cell autonomous components readily (although exceptions exist). Furthermore, questions have been raised about the likelihood of *ever* being able to use an iPSC approach for late onset diseases or for idiopathic forms of disease. Of course, a disease currently regarded as idiopathic may simply be caused by a complex set of genes whose expression is altered in a small number of patients, making genomics studies more difficult. Importantly, from the perspective of iPSC-initiated disease modeling, sets of mutations, however complicated, should be preserved in derived neurons.

For each class of disease, there are additional considerations: Which patient types have the highest probability of producing a robust, easy-to-study phenotype? Given patient-to-patient variability and additional complications surrounding the production of iPSCs and neurons, what are the most appropriate controls that will allow firm conclusions to be drawn? For obtaining robust phenotypes, choosing patients with the earliest onset, most severe pathology or most rapid disease progression seems to be a rational choice. Surprisingly, the topic of best controls is somewhat contentious. In the first few years of stem cell-based disease modeling, the standard approach was to compare phenotypes observed in a (very) small number of patient lines, often, but not always, from patients with known mutations, to an equally small (or smaller number) of controls. More recently, a preferred approach has been to start with patient iPSCs and use genome-editing technology to produce isogenic

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mutation-corrected lines to use as controls. Alternatively, some investigators favor the notion of introducing disease-associated mutations *into* control iPSC lines. While each of these has advantages and disadvantages, using multiple starting lines still seems advisable to get around idiosyncratic features of individual starting cell populations.

What Has Been Learned Thus Far?

Logically, for diseases with well-recognized later stages, it would make sense to reproduce the known pathology and then "rewind the clock" to determine the events that accompany disease onset. Thus, starting with AD patient biopsies, it would be exciting to produce neuronal cultures replete with plaques and tangles and with readily detectable synaptic dysfunction. Similarly using PD patient biopsies, it would be an excellent accomplishment to produce cultures of dopaminergic neurons that show accumulation of α-synuclein-containing Lewy bodies followed by selective neuronal death. To date, this has rarely been accomplished. Below, I summarize key features of some disease modeling studies that have been published in the last few years.

Spinal Muscular Atrophy

One of the seemingly simplest of neurological disorders is SMA, a childhood motor neuron disease caused by a mutation in the gene *survival motor neuron1* (*SMN1*). Several years ago, in one of the first disease modeling papers, Svendsen and colleagues produced an iPSC line from a skin biopsy taken from a child with SMA and showed that derived motor neurons had diminished survival (Ebert et al. 2009). Motor neuron death is, of course, an important feature of SMA. More recently, Corti et al. (2012) found reduced survival of SMA patient-derived motor neurons and showed that the defect could be corrected by genome editing. In both cases, motor neuron death occurred within one to two months or so, suggesting that onset of disease may have accelerated in the cell culture setting. Obviously, if this were to hold generally true, it would be very helpful to the disease modeling community.

More recently, my lab produced iPSCs from multiple SMA patients with mild to severe phenotypes (Rubin et al., unpublished). The first thing we did was to compare several different methods of reprogramming, including Sendai virus, episomal and modified RNA techniques. Reassuringly, results obtained comparing iPSCs, as well as motor neurons, were similar using all of the methods. Of extreme importance, the degree of pathology or abnormality that we observed in individual lines was directly related to the severity of the disease in the children who provided the biopsies. A big surprise was that the iPSCs themselves had a phenotype: in general, they had reduced proliferative capacity and were biased toward premature differentiation. This led us to predict that children with severe cases of SMA will have defects in other tissues. In

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fact, my laboratory previously showed that there is a similar proliferation/differentiation abnormality in muscle stem cells isolated from a mouse model of SMA (Hayhurst et al. 2012). An increasing number of other observations made either in children with SMA or in a mouse model of severe SMA indicates that there will be other tissue defects, as we are suggesting. This reinforces an important use of iPSC-focused studies: namely, the discovery of currently unknown disease symptomatology.

We also observed changes in motor neuron survival, but with some additional features. First, we found that neurons, in general, and motor neurons, in particular, differentiated faster and with greater efficiency when produced from mouse or human SMA ES cells or iPSCs. However, many of the prematurely born motor neurons died quite rapidly. Intriguingly, SMA is characterized by an acute phase followed by a more chronic phase, so this aspect of disease progression seems to have been maintained in the *in vitro* environment. Furthermore, at the single cell level, individual motor neurons with low protein levels of SMN died before those with high SMN. Conclusions from this work suggest that very early therapeutics for SMA (earlier even than currently thought) have the best chance of keeping the highest number of motor neurons alive and that SMN is a more precise regulator of motor neuron survival than has been previously suspected.

Amyotrophic Lateral Sclerosis

ALS is another motor neuron disease, but it is quite different from SMA. It is primarily idiopathic, but perhaps 20% of the cases can be accounted for by mutations in one of many different genes, including *SOD1*, *TDP43*, and *c9orf72*. It is also late onset and involves both upper (cortical) and lower (spinal cord) motor neurons. Nonetheless, it was one of the earliest diseases modeled using a ES cell approach. Experiments carried out using cultures of mouse spinal cord motor neurons made from wild-type ES cells and from ES cells expressing an *SOD1* mutation supported the idea that the disease has both cell autonomous and noncell autonomous components (Di Giorgio et al. 2007; Nagai et al. 2007). Specifically, motor neurons expressing the mutant gene were more likely to die over a two- to three-week culture period than control motor neurons. However, the death was again accelerated when motor neurons were grown in the presence of mouse astrocytes expressing the *SOD1* mutation (although, admittedly, the astrocytes were prepared as primary cultures). The negative effects of mutant glia could also be observed on human motor neurons. While some of these results had already been suggested by studies using transgenic mice, observations with cultured cells were especially convincing and provided a system that might, in principle, allow characterization of the toxic astrocyte factors. These studies had two important achievements. First, they showed that a feature of ALS (a late onset disease) could be observed when starting from stem cells, and that death was significantly faster than what

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is observed *in vivo* (mice from which the mutant ES cells were obtained die after about four months). Second, the variables involved in regulating the progression of disease could begin to be dissected. Several laboratories are also attempting to produce upper motor neurons from patient iPSCs, and it will be important to see if they can also duplicate disease phenotypes. Furthermore, there may be autoimmune or inflammatory components in this disorder that can perhaps be studied in iPSC systems.

My laboratory used wild-type and ALS mutant ES-derived motor neurons in a first of its kind screen (Yang et al. 2013). We tested about 5,000 small molecules to identify those that could promote the survival of both wild-type and mutant motor neurons. Our goal was to identify drugs that might act both in familial and sporadic forms of the disease. From our analyses, we identified multiple hit classes as well as a new drug target (MAP4K4) for treating ALS. We then showed that one of our most potent compounds was able to improve the survival of human motor neurons representing several different familial forms of the disease, all produced from iPSCs. By way of comparison, we also tested two drugs that had recently failed in Phase III clinical trials. Neither had been tested on human motor neurons prior to entering the clinic, but both had shown at least some efficacy in the mutant *SOD1* mouse model. One, dexpramipexole, was totally inactive on human motor neurons, while the other, olesoxime, showed inconsistent activity across different lines of motor neurons. This work suggests that (a) it is possible to carry out drug discovery screens at reasonable scale, even using motor neurons; (b) valuable information may be gained by pretesting ALS clinical candidates on human motor neurons; and (c) different forms of ALS may respond differentially to individual compounds, and this system may be able to help choose patients who are most likely to benefit from particular types of treatment.

Psychiatric Disease

More recently, scientists have begun to take on the challenge of using iPSCs to study human psychiatric disease, such as autism spectrum disorders and schizophrenia. Although these conditions may be diagnosed at relatively early ages, they encompass mutations in many different genes, each mutation occurring in a small percentage of patients. Environmental contributions seem likely as well. There is a significant debate as to whether there are multiple different forms of these diseases, such that each form may need to be treated with separate cocktails of drugs. Alternatively, many of the genes known to dysfunction are involved in synaptic transmission; thus, identifying a small set of common treatments may also be possible. This presents a particularly good opportunity to compare a genomics-based analysis to an iPSC-derived phenotypic method of study. In cases of extreme disease complexity, but with an underlying albeit complicated genetic basis, stem cell disease modeling might enable the

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emergence of robust and consistent disease phenotypes. Mapping these phenotypes back to the disease itself could, however, prove quite challenging.

A few have attempted to do so. Brennand et al. (2011) produced iPSCs from four schizophrenic patients, three of whom had a family history of disease, but none of whom had identified mutations. They used normal iPSCs as controls. Examining heterogeneous cultures of cortical-type neurons, they found that a consistent phenotype arose, one of reduced synaptic connectivity, not necessarily accompanied by changes in synaptic function. These defects could be partially corrected by treating cells with the antipsychotic drug loxapine. They also measured alterations in neurite growth, in the levels of some synaptic components and in the expression of particular gene pathways. They concluded that an iPSC approach might be revealing, even with such a challenging disease.

In a somewhat related study, Muotri and colleagues studied Rett syndrome, a genetic form of autism caused by mutations in the *MeCP2* gene (Marchetto et al. 2010). They compared heterogeneous cortical cultures prepared from iPSCs of four Rett patients to cultures prepared from control patient iPSCs. They obtained evidence of structural and functional alterations in the Rett cultures, which were partially correctable following treatment of cultures with IGF-1. Dolmetsch's laboratory studied Phelan-McDermid syndrome (Shcheglovitov et al. 2013), an autism condition associated with heterozygous deletions of chromosome 22q13.3. Again they produced iPSCs, in this case from two patients, and cortical neurons from the iPSCs. They observed synaptic defects, particularly in glutamatergic neurotransmission. These defects could be corrected by treating the cultures with IGF-1, but also, importantly, by expressing the *SHANK3* gene in neurons. *SHANK3* is one of the genes with previously suspected disease relevance in the deleted chromosomal region. Thus, Shcheglovitov et al. (2013) illustrate the progress that can be made even when studying patients with rather large chromosomal alterations (admittedly under circumstances in which there was a good candidate gene).

Parkinson Disease

PD is a classical late-onset disease characterized by protein aggregation (often in the form of Lewy bodies) and death of dopaminergic neurons. It is also thought that environmental factors, such as exposure to insecticide-like molecules which affect mitochondrial function, may be involved. Several studies have used the iPSCs from PD patients. Most of the studies have used familial cases, especially ones with mutations in the gene for *LRRK2* gene, a kinase known to be mutated in both some familial and some sporadic cases of the disease. Nguyen et al. (2011) produced immature neurons from a patient with a *LRRK2* mutation and showed that they were sensitive to a fairly wide range of stressors. In other words, a disease phenotype was discovered, but the known pathology of PD was not reproduced. Therefore, the relevance of the enhanced stress sensitivity to PD remains unknown. In another study, Liu et al. (2012)

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also produced *LRRK2* mutant iPSCs from which they derived neural stem cells, which were themselves passaged multiple times to add a stress component, at which point the cells showed deformed nuclei. The investigators then showed that similarly aberrant nuclear morphology could be produced in neural stem cells produced from human ES cells with the *LRRK2* mutation knocked in by viral-mediated gene targeting. Importantly, the phenotype of patient neural stem cells could be corrected by preparing an isogenic control line or by using a small molecule inhibitor of kinase activity. Thus, there seems little doubt that the phenotype relates to the genotype. The overarching question is: Does the phenotype relate to PD in any meaningful way? To answer this, Liu et al. looked at postmortem tissue derived from a PD patient and found that a percentage of hippocampal neurons also had misshapen nuclei.

Both of these papers described phenotypic differences between patient and controls, mostly in the realm of stress sensitivity. This has actually been seen a number of times in different neurodegenerative disease iPSC studies. Since this cellular behavior does not relate to any known aspects of disease pathology, what is this telling us? It may not explain components of the human disease as it develops in its true CNS setting, but may simply be a consequence of exposing cells to "unnatural" *in vitro* environments. On the other hand, perhaps real insight can be gained here—pointing us in the direction of identifying new and early features of even late onset diseases.

A recent study from the Studer lab is instructive in this regard (Miller et al. 2013a). These investigators tried to accelerate artificially the aging of neurons by expressing progerin, a mutated form of the *LMNA* gene involved in Hutchinson-Guilford progeria syndrome. They did get some encouraging results, pointing in the direction of being able to observe changes in dopaminergic neurons more like those seen in human PD patients, and suggest that late-onset aspects of neurodegenerative disease can be reproduced by accelerating the aging or maturation of neurons.

Using Patient Neurons to Find Therapeutics

Once an adequately controlled phenotype has been identified and confirmed across a suitable number of patients, a screen to find therapeutics can be established. There are several different ways in which this might be done. The most routine, or at least the most conventional, is to identify a druggable target based on a molecular dissection of disease phenotype genes or pathways. Under ideal conditions, the target could be screened in its endogenous cell type, although there are other options. Hits from target-based screens should then be checked for their ability to correct disease phenotypes. Alternatively, the screens themselves could be phenotypic (i.e., aim to correct a defect, such as reduced survival, in the diseased neurons) without even knowledge of an individual target. The advantages and disadvantages of each approach are still being debated, but

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phenotypic screens, in particular, may be able to capitalize on the strengths of iPSC disease modeling by collapsing changes in multiple genes and processes into a small common set of correctable phenotypes.

Most often, screens like these would be carried out and hits confirmed using a single patient line, presumably one with the easiest to quantify phenotypic change. Confirmed hits would be tested on multiple lines to determine how broadly individual classes of compounds might act. In the case of small molecule drugs, optimization of compounds would proceed as usual. As mentioned above, final compounds of interest can be tested for efficacy on patient neurons. However, they can also be tested for safety on cardiac myocytes and hepatocytes derived from the same patient iPSC lines. Admittedly, this type of testing can only capture toxicological events experienced by isolated single cell types, but future efforts will add additional complexity introduced at the organ level. Compounds of most interest would be those predicted to be effective and safe when applied to the highest percent of diseased patients. Alternatively, particular compound classes may be chosen based on their predicted safety and efficacy toward an identifiable subset of diseased patients.

The Future of the "Disease in a Dish" Idea

A tremendous amount of technical progress has been made in establishing systems and approaches capable of improving our understanding of neurodegenerative disease, leading to more effective treatments and a more efficient pathway into the clinic. Ultimately, the ideas put forth here can only be validated definitively once these concepts are tested on patients. This is, admittedly, a large hurdle. Over the next few years, it seems likely that at least some compounds, including those discovered by conventional means, will be tested on human-diseased neurons *in vitro* prior to testing them *in vivo*. Thereafter, it is likely that targets and compounds identified entirely using the methods described herein will be tested in a clinical setting.

At the moment, one of the major sources of disagreement between stem cell biologists and hard core neuroscientists relates to the lack of complexity of the stem cell-derived cultures, and hence, their potential inability to model neural systems in a meaningful fashion. Which is better: studying circuits and behavior in the mouse or studying simple human systems? It is fairly obvious that both systems are necessary and that both will always need to be evaluated critically. However, it seems highly unlikely that the notion of using human neurons, in the ways described in this brief article, will prove totally without merit. Rather, a likely view of the future is that stem cell biology, systems biology, bioengineering, and genomics will combine to produce more sophisticated functional neuronal circuits, followed by methods that allow for interactions among tissues in different organs, to capture important elements of human disease.

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