Neurons from reprogrammed cells

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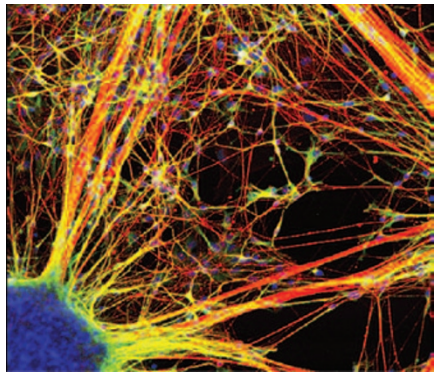
The potential is vast—but so is the uncertainty.

'Diseases in a dish' are starting to show intriguing symptoms. Neurons made from the reprogrammed cells of individuals with schizophrenia form relatively few connections, but these increase upon exposure to a known antipsychotic[1]. Neurons made from individuals with Parkinson's disease are more sensitive to oxidative stress[2] and show mitochondrial deficits[3]. Those made from individuals with spinal muscular atrophy die quickly in culture[4]. Those from people with Alzheimer's make higher concentrations of amyloid-β than those from people without the disease[5] (**Table 1**).

Not so long ago, these kinds of experiments would have been inconceivable. Compared with other organs, brain tissue is particularly hard to collect from patient biopsies. Neuroscientists have long had to make difficult choices between studying nonhuman cells or non-neural cells. But in 2007, researchers showed that human cells could be reprogrammed into a pluripotent state by adding genes active in the earliest stages of the embryo; the resultant cells could subsequently differentiate into all the major types of cells, including neurons. As reprogramming technology became more robust, academic hospitals around the world set up core facilities to generate cell lines from diagnosed patients. A cornucopia of induced pluripotent stem (iPS) cells has been derived from individuals diagnosed with myriad diseases.

Thanks to reprogramming, once-precious neural cells can now, in principle, be made in unlimited supplies. In addition, every cell line is genetically matched to someone with a known medical history. Human embryonic stem (hES) cells can also, in principle, supply endless quantities of cells. However, because making iPS cells is relatively easy and provides cells from a known individual for studying disease or developing therapies, much work on hES cells now involves using them as controls for comparison with iPS cells.

The potential of iPS cells has generated a steady drumbeat of publications. But, like all exciting technologies, the road from proof of principle to routine practice is arduous. Researchers hoping to use reprogrammed cells to study brain disease first have to deal with a host of less glamorous tasks: reducing experimental variation across cell lines, pro-



Neurons differentiated from induced pluripotent stem cells. Tubulin is red, dendritic marker MAP2AB is green and nuclei are blue. (Reprinted from ref. 1.)

Table 1 | Disease phenotypes observed in neurons made from reprogrammed cells

Disease	Mutations	Disease-related phenotypes	Types of differentiated cells	References
Alzheimer's disease[a] (adult onset)	Known contributing mutations	Altered processing and localization of amyloid precursor protein and increased concentration of amyloid-β	Central nervous system neurons	5
Amyotrophic lateral sclerosis	Known mutations	Altered cell signaling	Motor neurons	11
Familial dysautonomia (early onset)	Known causative mutations	Defects in gene splicing, neurogenesis and cell migration	Neural crest cells	7
Parkinson's disease (adult onset)	Known contributing mutations	More susceptible to cell stress Mitochondrial deficits	Midbrain dopaminergic neurons	2,3
Rett's syndrome (early onset)	Known causative mutations	Fewer synapses, smaller soma size, altered calcium signaling and electrophysiological defects Neural progenitor cells more susceptible to retroposons	Glutamatergic neurons	12–14
Schizophrenia (adult onset)	Unidentified	Fewer neurite outgrowths and less neuronal connectivity	Neurons	1
Spinal muscular atrophy (early onset)	Known causative mutations	Fewer cells in culture, synaptic defects, smaller cell bodies	Motor neurons	4

[a]Neurons from individuals with sporadic disease were made but not characterized. Source as indicated plus refs. 15,16.

ducing specific types and greater quantities of neural cells, and, most important, showing that phenotypes observed in culture are relevant for disease.

Neuroscientists are not yet ready to pronounce reprogramming a game changer in the study of brain diseases, says Margaret Sutherland, program director of the neurodegeneration cluster at the US National Institute of Neurological Disorders and Stroke. "Right now I would look at it as an additional tool," she says. "The full utility of these cells is still unknown."

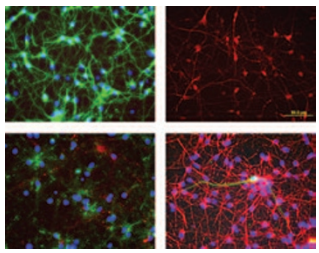
Insights from cultured cells

Having neurons to study in culture is "huge," says Deanna Benson, who investigates cells harvested from rodents at Mount Sinai Medical Center. "You can see things very easily." Unlike *in vivo* or *ex vivo* tissues, cultured neurons allow researchers to, for example, follow mRNAs in individual cells over time or track how protein synthesis within particular regions of cells is correlated with synapse formation. But after talking to many iPS cell scientists, Benson has no immediate plans to study neurons made from reprogrammed cells. "There's so much variability," she says.

When she collects tissue from, say, a developing rat hippocampus, she can be more confident about what the cells are. "When you take neurons out of their environment *in vivo* and put them in a dish, they still express their cell-type transcription factors," Benson explains. "They maintain their identity and continue to develop according to their origin." In contrast, reprogrammed cells that differentiate into neural cells can form mixtures of cell types that do not reflect their natural populations. "As a method right now for determining mechanisms of disease, [reprogramming is] not there yet," she says. "What [reprogrammed cells] are most useful for is trying to compare cells from a patient with a disease and, say, a family member who doesn't have the disease."

The importance of being human

Reprogrammed cells offer previously impossible opportunities to compare neurons from different individuals, experiments that could reveal genetic contributions to disease. Before iPS cells, researchers could study known disease genes by artificially introducing them into cells with viruses or plasmids. The approach works most easily for gain-of-function mutations, and distinguishing between effects of overexpression and mutation is difficult, says Sutherland. In



Induced pluripotent stem cells can be differentiated into neurons, as shown by multiple markers.

contrast, reprogrammed cells are expected to retain natural expression and can be studied even when the contributing mutations are unknown. New technologies are being developed to pinpoint meaningful sources of variation. Rudolf Jaenisch and colleagues at the Broad Institute recently used zinc fingers to engineer a specific Parkinson's mutation in iPS cells, creating cell lines that differ only in that particular sequence[6]. Eventually, such techniques could be used to insert putative disease mutations into cell lines to assess effects of a mutation.

For working with cells in culture, researchers can buy growth factors and other supplies for generating iPS cells and neurons from a wide range of companies, including BD Biosciences, Cayman Chemical, EMD4 Biosciences, Life Technologies, Millipore, PeproTech, Sigma, Stemgent, STEMCELL Technologies and Tocris Bioscience, just to name a few. Core facilities at academic centers also supply iPS cells, and the Coriell Institute for Medical Research holds fibroblast lines representing biopsies of dozens of individuals with neurological diseases. The NINDS human cell line repository at Coriell should soon begin distributing iPS cell lines from nearly 90 individuals representing disease and controls for amytrophic lateral sclerosis, Huntington's disease, Parkinson's disease and dementia.

Still, an overarching problem is making enough neurons. Other companies are focusing on supplying differentiated cells. Earlier this year, Lonza and California Stem Cell Technology began selling motor neuron progenitors derived from hES cells. Later this year, Cellular Dynamics International plans to launch a product called iCell Neurons, cells differentiated from iPS cells to form a consistent mixture of predominantly forebrain neurons (approximately half GABAergic, approximately half glutamatergic, with a smattering of dopaminergic neurons). Cellular Dynamics can prepare

single shipments of cells representing the equivalent of four percent of a human brain, a sufficient quantity to screen several thousand compounds in standard 384-well plates, says Brad Swanson, director of product development. And unlike cultures typically produced in academic labs, more than 95% of the cells are neurons, with very few cells expressing markers characteristic of neural progenitor cells, he says.

"Don't underestimate the importance of being able to produce these in quantity," says Chris Parker, chief commercial officer of Cellular Dynamics International, which supplies cells for drug screening and tissue engineering. Though the cells are not derived from patients with specific diagnoses, many relevant pathways can be monitored and manipulated. For example, says Parker, pharmaceutical companies are particularly interested in cells that allow analysis of how small molecules affect tau and amyloid-β expression and signaling. These proteins are implicated in Alzheimer's disease, but relevant signaling pathways are missing or different in rodents. In addition to its iCell Neuron product, the company also provides custom services, reprogramming and differentiating cells supplied via pharmaceutical companies or academic centers.

Questing for differentiation

Often differentiated neurons from individuals with brain diseases are not obviously different from those from healthy controls. Moreover, because cell lines show considerable variation, many lines are necessary to distinguish signal from noise. The current rule of thumb is to use at least three iPS cell lines from each affected and unaffected individual. Ideally, the individuals are related, minimizing genetic differences unlikely to contribute to disease.

Experiments are expensive and time consuming, says Clive Svendsen, director of the Cedars-Sinai Regenerative Medicine Institute. Making high-quality cell lines costs roughly $10,000 per individual and takes months, even with the efficiencies of an academic core center. In general, researchers test several lines per patient to weed out abnormalities that might be due to the reprogramming process rather than to an individual's genotype. Once characterized, lines from as many individuals as possible are differentiated and compared.

Differentiation itself requires costly reagents, particularly the highly purified proteins known as growth factors. A 100-day protocol that coaxes iPS cells into cortical neurons could easily run $1,000, Svendsen says. Thus, it is too costly to study as many cell lines as the number of animals typically used in *in vivo* studies, even though humans have far more genetic variation than strains of laboratory animals. Researchers must also be on their guard for differences that are due to artifacts from culture, he says. "The question is not really whether there is a phenotype," says Svendsen, "but whether it's relevant to the disease."

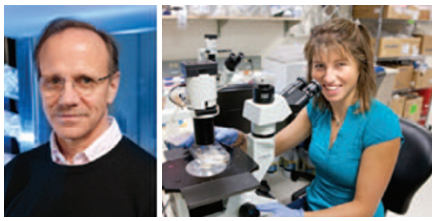
Lorenz Studer at the Sloan-Kettering Center for Stem Cell Biology is developing protocols to make specific subtypes of neurons.

One way to find phenotypes is to run cells through a battery of comparison tests. Ideally, though, researchers would start experiments with an idea of what types of phenotypes to expect. "It is important to have a clear idea of what to look for so you can ask precise questions," says Lorenz Studer, who directs the Sloan-Kettering Center for Stem Cell Biology.

So far, researchers have had the easiest time finding phenotypes for childhood diseases of the peripheral nervous system with clear causative mutations. For example, in the disease familial dysautonomia, blood cell lines derived from afflicted children have very low expression of the malfunctioning gene, hindering drug screens[7]. Differentiated neural cells, in contrast, show high expression. Even for diseases that typically strike in the brain during adulthood, inherited forms that manifest earlier in life are more likely to exhibit phenotypes. Researchers like Studer are searching for ways not just to create subtypes of neurons but also to 'age' neurons in culture, so that cells are more like those found in a septuagenarian and less like those in a newborn baby. The challenge is that, *in vivo*, it is unclear whether these neurons are defective from the beginning or whether the environment changes them during aging.

Seeking a subtype

Because brain diseases often affect particular types of cells, researchers are eager for homogenous populations of neurons that produce particular neurotransmitters or represent specific brain regions. When phe-



Fred Gage and his postdoc Kristen Brennard at the Salk Institute used differentiated neurons from people with schizophrenia and showed that they form fewer connections.

notypes are identified in cell types other than those responsible for disease, Studer says, it is hard to know how relevant a phenotype is. All cells in an individual with Huntington's carry the same mutation, but the striatal neurons in the center of the brain are lost in the greatest numbers as disease progresses, so studies in spinal cord neurons might not be the most enlightening.

Getting specific neural subtypes is a tall order. For example, dopaminergic neurons include light-sensing cells in the retina, extended cells in the substantia nigra and interneurons in the striatum. Even neurons existing side by side in the same region may differ in the peptides that colocalize with and regulate dopamine. The number of subtypes has been estimated in the hundreds. Even if a protocol has been reported, it may lead to less than ten percent of the desired neurons, says Sutherland. To create subtypes robustly, every aspect of production must be considered, says Studer. For example, prolonged exposure to fibroblast growth factor, which helps neural stem cells proliferate, can limit cells' ability to become forebrain neurons.

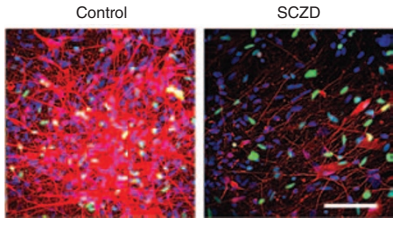
Difficult as it is, the production of specific subtypes is in high demand. "In the first phase of this work, the bar was lower," says Fred Gage, chair for research on age-related neurodegenerative diseases at the Salk Institute. It was enough to say what percentage of various neurons were present in a culture without pinning deficits on a specific population. Now researchers want to assess differences between subtypes in a disease context, and that requires making more specific types of neurons more efficiently. "These are really time consuming, difficult and expensive experiments," Gage says. "You have to figure out the series of growth factors and substrates to take the cell through the states so that it will give rise to those specific types of neuron. It's a science in itself."

The ability to make neuronal subtypes influences which areas are studied. Gage, for example, is primarily interested in neurogenesis within the forebrain, but when he first decided to explore whether differentiated neurons could be used to model disease, he chose amyotrophic lateral sclerosis, which affects neurons that stretch from the spinal cord to the muscles. "We started with the motor neuron protocol because it was there," he says.

Another challenge is knowing what type of cells have been created. To test a neuron's identity, researchers examine the markers cells express, evaluate whether cells can receive and transmit action potentials, and assess the type of neurotransmitters they produce. Other tests include profiling the entire transcriptome on very limited numbers of cells; a more rigorous test engrafts neurons in mouse embryos and tests their ability to function and integrate in developing tissue.

Creating specific types of neurons relies on a labor-intensive assessment of morphology and markers throughout the differentiation process, says Gage. If a cell-specific marker is on the cell surface, cells can be imaged and sorted with labeled antibodies. But many cell-specific proteins are internal, and so assessing these require additional steps. For example, the sequence of a promoter for a cell-specific gene can be fused with a reporter protein and stuffed into a lentivirus to label cells expressing that gene. One drawback is that these vectors can only hold about 7 kilobases of DNA, forcing researchers to guess which parts of a promoter are relevant. Bacterial artificial chromosomes can insert 200 kilobases but require considerably more labor to implement.

It would be far more desirable to encode the reporter genes into the cell lines themselves, says Svendsen, but human cells resist such engineering: successes using adeno-associated virus, zinc finger nucleases and transcription activator–like effector (TALE) nucleases have been reported,



Rabies virus modified to produce red fluorescent protein can be used to analyze connectivity of neurons made from iPS cells. SCZD, schizoprenia. Scale bar, 2 μm. (Reprinted from ref. 1.)

but efficient protocols are still being worked out and their use is not widespread.

Making cells and getting controls

In addition to the challenges of differentiation, researchers are trying to figure out the best ways to reprogram cells in the first place. Most recently, researchers have been working out ways to skip the iPS cell state with direct reprogramming, which replaces the transcription factors used to make pluripotent cells with transcription factors associated with a particular cell type. Just last year, researchers at Stanford University created neurons directly from cultured human skin cells, skipping the pluripotent state[8]. In September, the technique was used to make a specific neural subtype, spinal motor neurons, suggesting a way around the development of complex differentiation protocols[9].

"The question is not really whether there is a phenotype, but whether it is related to the disease," says Clive Svendsen at Cedars-Sinai Medical Center.

Direct reprogramming is still inefficient, and because it does not produce immortal cell lines, the cells produced are harder to bank and distribute. However, the technique can produce neurons in less time and with fewer expensive growth factors. But saving time and money is not the main advantage of direct reprogramming, says Asa Abeliovich at Columbia University Medical Center, who made neurons by directly reprogramming fibroblasts generated from individuals with Alzheimer's[5]. Because iPS cells have been selected for their ability to grow and form colonies, these populations are more likely to show genomic instability and tumorigenicity than are directly reprogrammed cells, he says. And direct reprogramming may offer still more advantages. "Though it remains to be demonstrated," Abeliovich says, "directed conversion of human skin cells to neurons cuts out the iPS middleman, and thus may generate cells that more closely reflect human disease states."

Whatever the reprogramming technology, having a desired subtype—interneurons for schizophrenia or striatal neurons for Huntington's—will not guarantee cells that can supply the answers. Too much biology is unknown. Sometimes, it is unclear which cells to examine in the first place. In an early proof-of-principle study showing that differentiated cells could be used to model human diseases, Gage cultured human astrocytes carrying a mutation linked to amyotrophic lateral sclerosis (ALS) together with motor neurons that had been differentiated from hES cells. Although the motor neurons die in the disease, results indicated that the fault was not in the motor neurons themselves but rather in the astrocytes that support them[10].

Perhaps the most difficult issue is that researchers have few ways to know whether their results are valid when studying human disease, particularly late-onset disease for which no effective treatments are available. The problem exists at several levels: if reprogrammed cells are made using a biopsy collected from an undiagnosed 40-year-old, there is no way to know whether that individual will be healthy at 60. Deficits that cells show in culture may not reflect what happens *in vivo*, and vice versa. In cardiovascular disease and cancer, says Svendsen, researchers can often compare screened molecules to drugs that have already demonstrated some benefit in humans, but such resources are often unavailable for testing differentiated neurons. "We're woefully inadequate for drugs that work in the brain," says Svendsen. "But maybe," he adds hopefully, "we don't have the drugs because we've never had this model before."

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