Between disease and a dish

Neuron- and glia-like cells derived from induced pluripotent stem cells promise tractable, individualized human models of Alzheimer's, Parkinson's and amyotrophic lateral sclerosis. Ken Garber explores the considerable challenges of recreating such diseases in the laboratory.

The use of induced pluripotent stem cells (iPSCs) for disease modeling is spreading widely, with no area more fertile than neurological disease. At the 2013 annual meeting of the Society for Neuroscience in San Diego, last November, 57 posters presented data on modeling 24 different neurological diseases using iPSCs, including 12 models of Parkinson's disease alone. Technical ease is one reason for all the activity. Reprogramming fibroblasts into iPSCs and differentiating them into neuron-like cells does not require expensive equipment or exotic skills. Mahendra Rao, who in April moved from the recently shuttered US National Institutes of Health (NIH) Center for Regenerative Medicine, has pointed out that "a small lab [that] can do standard biochemistry or do standard molecular biology can make iPS cells…It's really, truly technology for the masses."

Another factor is the need for better models, given our poor understanding of the pathogenesis of complex diseases and the dismal predictive record of animal models and tumor-derived cell lines. Patient-specific human cell lines seem an ideal platform for mechanistic studies and drug screening, and proponents of iPSCs have hyped their promise almost from the moment of their discovery, in 2006. But superiority to existing models is not yet evident. "They need to demonstrate their worth," says Rick Livesey, a Cambridge University neuroscientist. "We need to test the rhetoric that we've set, that these are inherently more useful in some ways than animal models, because you're doing it on the background of the human genome and the human proteome."

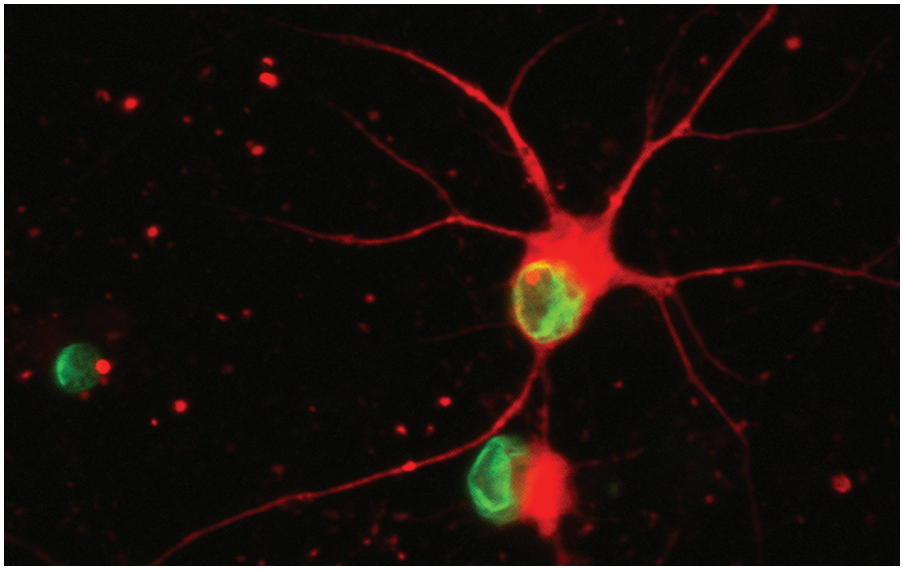
Nowhere is that need more urgent than in common neurodegenerative diseases. For example, Alzheimer's and Parkinson's together afflict about six million people in the US alone. So far, most of what has been learned from iPSC-based models is that they're technically feasible. But they're still a long way from the ideal of human disease in a dish.

Plumbing a mysterious ALS mutation

Amyotrophic lateral sclerosis (ALS) is proving an excellent test case for iPSC disease-modeling technology. How the disease causes cell death and why it selectively targets motor neurons remain unknown. One clue came, however, in 2011, when geneticists discovered that much of familial ALS, which represent roughly 5% of all ALS cases, was due to one disease-causing mutation, a repeat sequence in the C9orf72 gene. The finding has captivated the ALS field because the mutation is so common, not only accounting for 40% of familial ALS, but also causing at least 7% of sporadic ALS cases. (It plays an even bigger role in frontotemporal dementia, or FTD.) Successfully modeling C9orf72 ALS could have a big impact on ALS and FTD research without taking on the more intractable problem of sporadic disease. And because the mutation is extremely hard to reproduce in transgenic mice, as the massive expansion sequence can't be easily cloned into mouse embryos, iPSCs are an obvious approach.

None of this has been lost on the field. Over a 16-day span in October 2013, three separate groups published papers modeling C9orf72 ALS in human cells, two using iPSCs[1,2]. And this is just the beginning. "These papers have started what will be an avalanche of work," predicts Richard Wade-Martins, a molecular geneticist at the University of Oxford, UK.

To some extent, the recent iPSC papers vindicate the technology. Neurons derived from these iPSCs display a key C9orf72 phenotype: abnormal protein–RNA deposits or inclusions, called nuclear foci, containing the RNA repeat. And antisense oligonucleotides from Isis Pharmaceuticals in Carlsbad, California, targeting the repeat, suppress the nuclear foci. "We're all finding the same thing," says Jeffrey Rothstein, a neurologist at Johns Hopkins University in Baltimore, and senior author on one of the papers: "That there are inclusions in the human cells [and] with the right antisense targeting the expansion you can get rid of these inclusions."



Lorenz Studer, Memorial Sloan-Kettering Cancer Center, New York

Stem cell–derived nerve cells exposed to progerin to induce aging. The top right cell is still healthy; the cell below is losing its processes.

These experiments also go some distance toward debunking one theory of C9orf72 function, that the mutation impairs the activity of C9orf72, whose function is unknown, causing the disease. In cells, progressive knockdown of C9orf72 didn't increase markers of disease severity; it actually made the cells healthier. This cell line evidence "is pretty damning to loss of function" causing the disease, says Rothstein.

The models could solve another puzzle. A leading theory for how the C9orf72 mutation causes ALS is that the repeat generates an abnormal RNA structure that traps RNA binding proteins, which build up in the nuclear foci. This prevents them from carrying out their normal RNA processing

function in neurons, which then die. The identity of the sequestered protein or proteins is a much sought-after prize in the field. In the iPSC-derived neurons of both recent papers[1,2], the authors identified the RNA-binding proteins bound to the RNA repeat. Unfortunately, they weren't the same proteins, so the results await replication by others.

There were other problems. Strikingly, neither group found certain basic features of ALS pathophysiology in their cells. For example, inclusions of TDP-43, an RNA-binding protein, are the pathological hallmark of ALS and FTD. As yet, these inclusions have not been observed in iPSC-based models. "They're definitely not there," says Robert Baloh, a neurologist at the University of California, Los Angeles, and senior author on one of the papers[1]. "And really, the cardinal feature of motor neuron disease is motor neuron degeneration, right? I mean, the cells die. And that didn't happen either."

Lacking such basic ALS features in these cells, the models are suspect. Some investigators speculate that the discrepancies might be due to iPSCs not differentiating into a cell resembling the right motor neuron subtype. (Some subtypes may be more vulnerable to ALS than others.) Alternatively, the cells may have been harvested at an intermediate level of development without having reached full maturity. Several groups are deriving astrocyte-like cells from ALS patient iPSCs because astrocytes are involved in the disease. (ALS astrocytes can kill wild-type motor neurons.) The same subtype and maturation concerns that apply to neurons apply here as well.

But the main obstacle, Baloh ventures, may be cellular aging—or the lack thereof. Cytoplasmic TDP-43 and neurodegeneration have long been observed in ALS autopsy samples. But because reprogramming appears to reset cells chronologically, they're modeling "a much earlier phase of the disease," says Baloh. "We're probably looking at the presymptomatic phase." To begin seeing cytoplasmic TDP-43 and cell death, researchers may have to induce aging. "It's really the quest to create a much more realistic model," says Baloh. "Develop the most mature-looking neuron and then try to either age it in culture or incorporate stressors…that would enhance or mimic the aging process." The goal would be to bring out more ALS phenotypes. "That's one of the primary goals of all iPS cell disease modeling, at this point, for any disease," says Baloh.

Young versus old cells: which are better?

Among the common neurodegenerative diseases, Parkinson's is by far the most popular for modeling using iPSCs. At least 12 different groups have published Parkinson's models since 2011. Interest partly owes to the uniqueness of the cell type, the midbrain dopamine neuron, which bears the brunt of disease pathology. Snaking its unmyelinated axons all the way from the midbrain to the forebrain, creating massive axonal arbors that form hundreds of thousands of synapses, each cell also fires regularly, like a pacemaker, as it orchestrates the release of dopamine. As a result, energy demands are enormous and dopaminergic neurons are vulnerable to the slightest disruption. "The question is not so much why they die in Parkinson's, but how they stay alive in the first place," says Wade-Martins.

Parkinson's is attractive for modeling in other ways. As in ALS and Alzheimer's, modeling the rare familial forms of the disease is far more informative because the genetic component is well-defined, usually highly penetrant, and acts early in the disease. But, in Parkinson's, these studies should also shed light on sporadic disease because genome-wide association (GWAS) studies on risk factors have implicated many of the same genetic loci as are found mutated in familial Parkinson's. Parkinson's disease mechanisms are also better understood than those of ALS and Alzheimer's, so many relevant assays are available to researchers.

Genetic, biochemical and pathological studies have implicated mitochondrial dysfunction, not surprising in such metabolically active cells. Energy deficits lead to an inability to deal with protein turnover, to defective autophagy and eventually to cell death. Most iPSC modeling to date has confirmed these mechanisms. "Findings are coalescing around mitochondria, ER [endoplasmic reticulum] stress, UPR [unfolded protein response], autophagy and lysosomes," says Wade-Martins. For example, a 2012 study by a group led by Ole Isacson, a neuroscientist at Harvard University's McLean Hospital in Belmont, Massachusetts, showed that neurons derived from the cells of patients with PTEN-induced putative kinase 1 (*PINK1*) mutations, which cause familial juvenile Parkinson's, and leucine rich repeat kinase 2 (*LRRK2*) mutations, powerful Parkinson's risk factors, had altered oxygen consumption (*PINK1* higher, *LRRK2* lower), compared with nondisease cells, suggesting distinct forms of mitochondrial dysfunction[3]. Both Parkinson's mutant cell types were also more sensitive to chemical stressors. "In both cases, when we challenged the cell, they had severe trouble," says Isacson.

But Isacson's cells did not show alpha-synuclein aggregation, a hallmark of Parkinson's seen in autopsies, and the authors did not report a higher rate of cell death compared to healthy controls. The absence of these disease signatures does not surprise or trouble Isacson. "It's not reasonable to assume that Parkinson's disease will appear in an immature embryonic fetal neuron," he says, "unless we grow the cells for maybe 70 years." Instead of a liability, in Isacson's view, this lack of phenotype is an opportunity. In Parkinson's and other neurodegenerative diseases, he says, young iPSC-derived neurons offer a window on early disease pathology, which should help identify disease drivers. Isacson has described some disease pathology already present in these cells[4]. "The end product may be some protein folding problem later on, but the early form of the disease is probably distinct from the late phase…. And the late stage basically is the last effort the cell makes to prevent cell death."

Rather than trying to recreate late-stage disease in a dish to look for phenotypes, like alpha-synuclein aggregation, says Isacson, investigators should focus on early events. "I feel that one should stop looking under the streetlight and maybe use these cells to find new mechanisms, not just the ones we already know about," he says.

But others feel that old neurons are the more clinically relevant population. In familial Parkinson's, patients' cells bear the mutations from conception, yet Parkinson's symptoms only appear late in life. "These cells are perfectly capable of preventing the disease from manifesting itself early on, despite the same DNA," says Lorenz Studer, a neuroscientist at the Memorial Sloan-Kettering Cancer Center in New York. "If you really want to understand why they start to die at that late stage, then I think we need to have the ability to have those [aged] cells in our hands." Drug screening applications, he adds, should also employ aged cells. "Why would you perform a screen in a cell that's actually perfectly capable of suppressing the disease already?"

Researchers have tried to simulate cellular aging by applying chemical mitochondrial stressors or oxidizing agents. Studer recently reported a new method: exposure to progerin protein[5]. Progerin, the product of a mis-spliced transcript in the lamin A gene, causes Hutchinson-Gilford progeria syndrome, a rare disease resembling accelerated aging that leads to early death. After identifying a panel of molecular age-associated markers and finding that iPSC-derived cells do not retain a memory of age, Studer tried exposing mature differentiated midbrain dopaminergic neurons to progerin for five days. In addition to morphological and DNA damage, shorter telomeres and signs of oxidative stress, Studer observed a shrinking of the dendrite network, "exactly

Box 1 Understanding risk variants

For gene loci identified by GWAS studies, iPSC-derived cells can, in theory, model how these risk factors set the stage for disease. For example, an obvious approach is to co-culture astrocytes of one ApoE genotype with neurons of another ApoE genotype, and then observe the effect on disease progression, to isolate the exact cell type the pathogenic ApoE variant is acting in. Larry Goldstein's laboratory is now doing this. Rare mutations with large effect, like the risk variant in the *Trem2* gene, which encodes a membrane protein found on microglia, and the A673T protective variant in *APP*, will probably be other early projects.

Most GWAS loci are more problematic because "they don't point to a coding variant, they simply point to an association with a locus," says Rick Livesey. "You don't really have a hypothesis you can test." Also, individual effects may be too small to be detected over background noise. Goldstein, who is doing such experiments, is nevertheless optimistic. GWAS studies, he points out, "tell you what the average effects are. They don't tell you about individual effects. if you know how to focus in on a subset [of individuals], where you have the right measurements, you can resolve signal out of that noise."

what you would expect to happen in an aged cell," he says. [See photo.] Cells taken from Parkinson's patients with *PINK1* gene mutations had enlarged mitochondria, whereas cells with disease-causing *Parkin* (also known as *PARK2*) mutations showed "multilamellar" inclusions thought to be Lewy body precursors. "All the phenotypes that you would think are involved in aging, let's say telomere biology, let's say mitochondrial stress, let's say protein aggregation…are actually produced by progerin," says Studer. "It clearly phenocopies age remarkably well."

Isacson isn't convinced that progerin is the best way to induce aging in Parkinson's cells. "In no way does it actually simulate what I believe [are] the most common pathways for generating cell biology in Parkinson's disease," he writes in an e-mail. Mitochondrial DNA deletion, in Isacson's view, is the key hallmark of disease progression. But he thinks progerin may be useful in other diseases. Studer says the technique should be widely applicable.

Alzheimer's pitfalls

Fewer people work on Alzheimer's disease iPSC modeling than on Parkinson's, for one simple reason—Alzheimer's is a more formidable modeling challenge. To begin with, there's no consensus view on what constitutes the critical disease pathology, so it's hard to know whether a cell in a dish is accurately modeling the disease. Also, synaptic dysfunction is present in early Alzheimer's, but is difficult to model with iPSC-derived neurons. Unlike in Parkinson's, Alzheimer's GWAS studies (**Box 1**) have not implicated any of the familial disease mutations in sporadic disease, so modeling familial disease, while easier, may not be very informative for sporadic Alzheimer's. The most common risk variant is *APOE4*, and the APOE protein isn't even expressed in neurons (although it is in astrocytes). Finally, there is a clear immune system component to Alzheimer's, which will be complicated to model *in vitro*.

But there are laboratories committed to overcoming these problems, starting with the absence of ApoE4 in neurons. "If the issue is ApoE4…you can make astrocytes and study them," says Larry Goldstein, a neuroscientist at the University of California, San Diego. "If the issue is that purified neurons don't model the brain, yes, that's true. So you start to set up co-cultures with defined cell types. If the issue is we don't know what the precise phenotype is—yes, fair enough. You can, however, identify biochemical changes in the very few pathways that are acknowledged to be disease positive. Namely, changes in APP [amyloid precursor protein] behavior, changes in tau protein behavior." Researchers working in other diseases, Goldstein notes, have developed ways of measuring synaptic changes. "My labs and other labs working on this [iPSC] technology are in the process of trying to use those approaches to study synaptic maintenance in Alzheimer's disease cells," he says.

"You have to make progress logically and stepwise," Goldstein says. "So you start with simple systems and you gradually add complexity. And that's what the field is doing."

Goldstein's group and others already have reported some novel findings using neurons derived from reprogrammed Alzheimer's patient cells[6]. The cell type of choice is the glutaminergic cortical neuron because the cortex is the largest part of the brain affected by the disease, and the forebrain neuron is the virtual default pathway for stem cell differentiation.

Goldstein's group reported higher levels of beta-amyloid peptide, phospho-tau and glycogen synthase kinase (GSK)-3β, an enzyme that phosphorylates tau, in neurons derived from Alzheimer's patients. They also saw enlarged endosomes, which had earlier been reported in postmortem brains. In Goldstein's system, only β-secretase inhibitors, and not γ-secretase inhibitors, are able to reverse the tau and GSK-3β changes, suggesting that the C-terminal fragment of the amyloid-β peptide, which is generated by β-secretase cleavage of APP, is a bad actor in the disease.

Other groups have reported more than biochemical changes. In 2012, Livesey's group at the University of Cambridge used neurons derived from cells of individuals with Down's syndrome to model Alzheimer's disease[7]. Many adults with Down's develop early-onset Alzheimer's, and the Down's chromosome 21 duplication reproduces the APP gene duplication seen in Alzheimer's families. Livesey's group observed both intracellular and extracellular amyloid-β aggregates for the first time. They also described the release of phosphorylated tau from neurons, something also reported at meetings by the S. San Francisco, California–based biotech company Ipierian, which claimed to have identified a novel, cleaved version of extracellular tau[8]. Bristol-Myers Squibb of New York acquired Ipierian in April and hopes to launch a clinical trial of Ipierian's antibody targeting this "e-tau" next year.

"It's very early in the game," says Goldstein. "A lot of what's happening is technological. But…it's encouraging that some novel insights are emerging."

A fundamental goal of the iPSC field is to understand disease progression—how neurodegeneration spreads from cell to cell. For example, evidence is accumulating in Alzheimer's that transfer of tau between neurons plays a role in disease spread, but no one knows if this can be modeled in a dish without overexpressing tau. "We simply have to go look, and work out how long does it take, and at what level of expression of the protein does it happen," says Livesey. "Because we all want this to be physiological levels of expression."

Looming challenge

Finally, there is the enormous challenge of modeling sporadic disease and mining these models for useful biological insights. As of early May, cells from seven individuals with sporadic Alzheimer's had been reported reprogrammed and converted into cortical neurons, and four of these lines showed biochemical changes associated with the disease[6,9,10]. Four is barely a start. "It may be a bit of a numbers game," says Goldstein. "You have to have

Box 2 Maturity in a month?

Generating neurons from fibroblasts, by way of iPSCs, is a very slow process. It takes up to a month for reprogramming to yield colonies of iPSCs, then as many as 3months longer to harvest mature neuron-like cells. In the case of midbrain dopaminergic neurons, to model Parkinson's disease, "it happens only by about day 70 or day 80 of differentiation," says Lorenz Studer. "You need to be very patient to do these experiments. And that makes it much more complicated. And it's also technically difficult." For example, it's almost impossible to use iPSC-derived dopaminergic neuron-like cells for high-throughput drug screening because by the time they're functionally mature, they've grown elaborate axonal processes that prevent their plating on 384-well plates. Ripping off the processes usually causes cell death.

Direct conversion or transdifferentiation, which bypasses the pluripotent stage, is much quicker, but is less efficient and produces nonrenewable cell populations that have trouble forming synapses. (In 2011, a Columbia University group reported in *Cell* on functional neurons made directly from the fibroblasts of Alzheimer's disease patients, but the authors recently retracted the paper[11].)

Technology, meanwhile, is chipping away at the differentiation and maturation bottlenecks. Marius Wernig and Thomas Südhof of Stanford University have recently reported that forced expression of a single transcription factor could differentiate iPSCs into neurons in fewer than two weeks[12]. "That speeds things up a bit," says Rick Livesey, although it remains to be seen whether all neuron types can be produced this way. It should also be possible to accelerate the maturation process. In 2012, Studer's group reported that treating iPSCs with a cocktail of small molecules could generate functional nociceptive neurons in ten days[13]. Studer expects this approach to eventually work for other neuron types. "It's still a little bit tricky," he says. "With dopaminergic neurons, we are not completely ready. With cortical neurons, we have had some good success, very similar results, but still unpublished."

Studer cautions that taking these neurons to full maturity will take additional time. Still, he says, using small molecules could cut 3–4 weeks from the differentiation process. "It might make it possible to do a high-throughput screen all the way from a pluripotent cell to a neuron in a 384-well plate, in one shot, without ever replating," he says. (Leaving iPSCs on a plate for longer periods of time, without the ability to manipulate the culture system, leads to variation that confounds drug screening results.) Biology does set limits, however. If all the improvements pan out, the overall differentiation time "probably won't get much shorter than a month, I suspect," says Livesey. "Which still, if you put it in perspective, is pretty dramatic."

enough numbers of different genomes, with enough precision measurements, that you can test whether the genetic changes in these sporadic genomes lead to a consistently measureable phenotype."

Hundreds of cell lines will probably be necessary to begin to do meaningful biology for the sporadic form of any of these diseases. For Parkinson's, the US National Institutes of Health in 2009 sponsored a consortium to develop such lines, resulting in about 30 sporadic lines, says Isacson, who led the effort. "The opportunity will be when you reach two, three hundred lines," he says. The New York Stem Cell Foundation is partnering with a Parkinson's research foundation and with Mount Sinai Medical Center in New York on large- scale, cell-line production efforts in Parkinson's and Alzheimer's, respectively. In the UK, an academic–industry partnership, called StemBANCC, aims to generate 1,500 well-characterized cell lines from 500 individuals, with an emphasis on neurological disease, by 2017.

Researchers are already preparing to deal with the heterogeneous cell lines that will come from these patients with sporadic disease. Isacson expects that some cases of sporadic Parkinson's will closely resemble familial Parkinson's molecularly, and he's generating phenotypic "scorecards" from familial studies that can be used in the future to categorize sporadic disease. Studer thinks progerin-induced aging could speed this process, by making the sporadic disease phenotypes, especially the biochemical pathways that lead to cell death, more obvious. "We could then match them, for example, to the pathways that happen in any of the familial forms of the disease," he says.

There may be other ways to find order amid the likely chaos of sporadic disease. In Alzheimer's, Livesey notes, twin studies show the genetic contribution to Alzheimer's to be in the range of 50–75%. So it might be possible to identify individuals with known genetic risk factors whose cells can recapitulate the disease in a consistent way, and serve as models. "The problem there is, they'll replay the disease for you, but will you then be able to use them to unpick the actual disease process?" he says. "You might be able to get a lovely phenotype, but other than reproducing the phenotype, you may not have learned very much."

Reprogramming technology has passed the first test, generating neural cell lines that show biochemical changes compatible with known disease processes. Besides overcoming practical limitations (**Box 2**), researchers now must prove the usefulness of these models. "The field has done the simple phenomenology," says Livesey. "We've shown that you can make neurons, they're credible, they're not very different from what we would call a primary culture, that if you could isolate neurons from a human they would look pretty similar in the dish. With some question marks still over the maturity, but I think those are solvable. I think the big question is, can we do real biology with these things? I think we can."

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