IPSCs: One cell to rule them all?

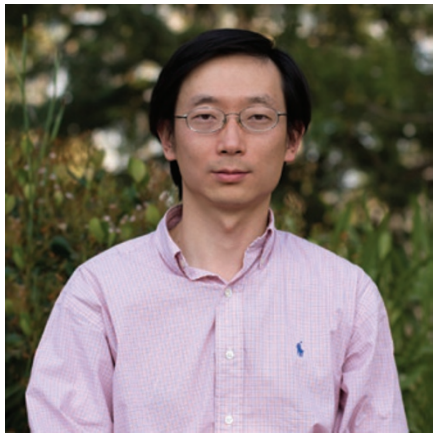
Michael Eisenstein

Rapid progress with induced pluripotent stem cells is bringing scientists closer to understanding their strengths and weaknesses as embryonic stem cell stand-ins.

In 2006, with formidable legal and technical obstacles keeping the promise of embryonic stem cells (ESCs) in check, Shinya Yamanaka's announcement was truly a scientific 'shot heard around the world'. He and his team at Kyoto University had reprogrammed adult mouse fibroblasts into so-called induced pluripotent stem cells (iPSCs)[1], opening the stem cell field to legions of eager scientists and offering the promise of unprecedented capabilities for targeted disease research using stem cells derived directly from patients of interest.

Yamanaka's approach of genetically induced reprogramming itself was not revolutionary, and the four reprogramming factors he identified (Oct4, Sox2, c-Myc and Klf4) were known to contribute to cell proliferation and maintenance of pluripotency; what was remarkable was finding a combination that actually worked, even at modest efficiency. "People have been working on reprogramming with nearly identical approaches and concepts for a long time," says Sheng Ding of The Scripps Research Institute, "but I would say that most attempts failed; that's why this work with iPSCs was a breakthrough discovery." Subsequent work by James Thomson and colleagues replicated Yamanaka's success with human cells and revealed additional factors—Nanog and Lin28—that facilitate the reprogramming process[2].

Just three years later, the field has exploded, and many of the tools for iPSC production, characterization and differentiation are now available 'over the counter' from a variety of institutions and companies. "When everyone was doing ESCs, there was that sense that each individual ESC line was precious," says Nick Seay, chief technology officer at Cellular Dynamics International (CDI). "The whole paradigm has shifted: there can be a line or three for everybody, and we're looking at everybody's stem cells."



Sheng Ding's team at The Scripps Research Institute is developing techniques for vector-free reprogramming of iPSCs. Courtesy of S. Ding.

Embedded instructions

Today, scientists can choose from a variety of commercially available systems, such as ArunA Biomedical's viPS vector kit or various Stemgent Reprogramming lentiviral and retroviral systems; these typically consist of a collection of vectors, each containing a gene encoding one of the various so-called 'Yamanaka' or 'Thomson' reprogramming factors.

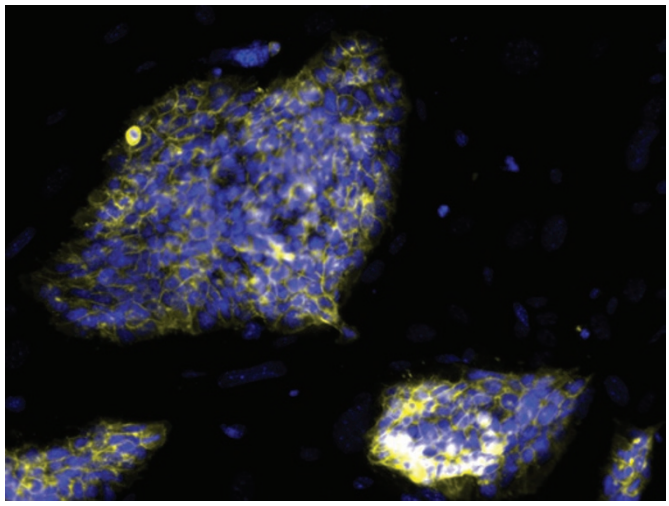
The requirement for multiple vectors can be somewhat cumbersome, and several groups have developed single-vector systems in which the coding sequences for the various factors are linked by self-cleaving sequences. In contrast, supplying each vector separately allows researchers to pare down the number of factors—a useful asset as investigators strive to identify the minimal set required for iPSC production. "People have shown that you can reprogram with variations: you can do three-molecule, two- or even one-factor reprogramming," says Stephen Chang, chief scientific officer at Stemgent.

One problem with trying to use vectors to dissect the reprogramming process is the relatively uncontrolled expression of the factors. Rudolf Jaenisch's team at the Massachusetts Institute of Technology has tackled this problem through the use of tetracycline-inducible promoters; this enables careful, drug-mediated control of gene activity and has helped his group to greatly improve their overall efficiency of iPSC production[3]. Reprogramming vectors based on this principle are currently available from Stemgent and Life Technologies.

Uninterrupted programming

A more serious issue pertains to potential damage from genomic disruption. The most-established techniques for iPSC production use retroviral or lentiviral vectors, which integrate into the genomes of reprogrammed cells and thereby become a lasting, problematic source of variation and unpredictability. "Viral integration in some genes causes potential for oncogenesis; certainly this has been shown in mice," says Chang. As such, a great deal of effort is now being invested into the development of 'self-cleaning' vectors that can be precisely removed from the genome once reprogramming is complete, and some alternatives under investigation include the use of transposons or site-specific recombination systems like Cre-*loxP*.

However, the most intense interest is in 'vector free' systems, in which no integration ever takes place, such as



iPSCs produced using Stemgent's Mouse iPSC Generation Kit, with stem cell marker SSEA labeled in yellow via immunofluorescence and cell nuclei costained using 4',6-diamidino-2-phenylindole (DAPI). Courtesy of Brad Hamilton, Stemgent.

adenovirus-based approaches or the BacMam system from Life Technologies. "BacMam are modified insect-specific baculovirus adapted for expression of transgenes in mammalian cells; they can hold large gene fragments and can transduce diverse cell types with minimal toxicity," says Mohan Vemuri, director of research and development for primary and stem cell systems at Life Technologies. "We have created BacMam particles containing individual and multiple reprogramming factors [that] have the potential to reprogram somatic cells without integration."

Thomson's team developed another alternate approach, using a non-integrating episomal vector derived from Epstein-Barr virus, which can be stably maintained in transfected cells via drug selection; after reprogramming, the removal of drug selection results in gradual loss of the vector during multiple rounds of cell division[4]. This method is currently in use at CDI, a company cofounded by Thomson. "When we're done with the cell, its genome is pristine," explains Seay. "The episomal method we have appears to work across a variety of cell types, and it seems that we can get it in an efficiency range where, in the near future, we'll be able to readily make [iPSC] lines from samples from patients."

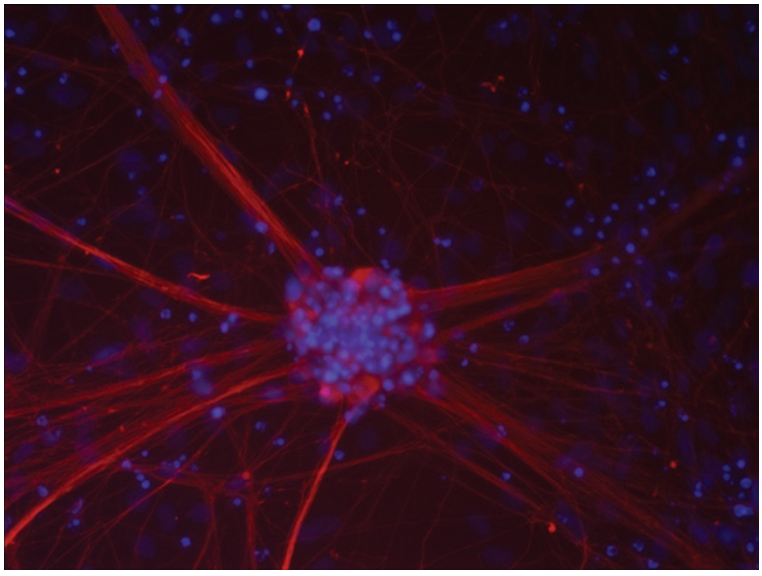
One promising strategy forgoes genes altogether, relying instead on direct delivery of the reprogramming proteins themselves. This method, developed in a collaboration between the Ding laboratory and protein production specialists at ProteomTech, uses modified versions of the various reprogramming factors that have been tagged with specially designed charged domains that can bind to plasma membranes and facilitate protein entry into cells and, ultimately, into the nucleus[5]. Such chemically defined techniques are not only suitable for wider applications but are also easier, says Yong Zhu, vice president of Research and Business Development at ProteomTech. "If you want to use viruses or other approaches, you have to be an expert in the method." The company is currently working to optimize and commercialize its protein-based reprogramming approach.

Accident or design?

Right now, though, most researchers making iPSCs are sticking to the standard viral techniques. "If you just want to study reprogramming in the lab or if you're doing disease modeling in iPSCs, then retrovirus is basically fine," says Holm Zaehres, of the Max Planck Institute for Molecular Biomedicine. These are, after all, more efficient than the best nonintegration-based techniques by at least an order or two of magnitude—and even the integration-based techniques have considerable room for improvement.

According to Ding, more systematic techniques are the key to boosting efficiency: "The current iPSC reprogramming process is essentially an undirected, nonspecific process involving so many stochastic, random events," he says, adding that "ultimately, you want to have a directed reprogramming process to obtain iPSCs, to make the process more efficient and robust." Members of his lab have made considerable progress on this front by exploring the capabilities of small molecules to improve the speed and efficiency of reprogramming and the maintenance of pluripotency. In recent work, Ding's team described how the addition of three small-molecule compounds—two signal transduction inhibitors and a chemical that promotes cell survival—boosted reprogramming efficiency more than 200-fold[6].

A growing number of other small molecules are finding widespread use as reprogramming supplements, such as valproic acid, a compound that contributes



Scientists at iPierian are using motor neurons and glia derived from human iPSCs to study neurodegenerative disorders. Courtesy of Ashkan Javaherian, iPierian.

to epigenetic reprogramming as a histone deacetylase enzyme inhibitor. Stemgent is among the companies working to make such chemical tools commercially available as soon as possible, and Chang says that he expects these small-molecule discoveries to continue.

Increasingly, experimental evidence suggests that such molecules can replace some reprogramming factors entirely. Ding believes that, in one to three years, a chemically defined small molecule cocktail will be available that can be used to generate iPSCs without genetic manipulation or even proteins. This would make iPSC production more consistent and more convenient, he says, adding that with this approach "you have more control, and chemicals are more stable, easier to manufacture and a lot cheaper."

Know your stem cell

Though many tools to cultivate ESCs and iPSCs are interchangeable, manufacturers are continuing to test and optimize culture reagents expressly for iPSC work; for example, Stemgent is preparing to release a new medium that helps to drive dedifferentiation by establishing culture conditions that facilitate epigenetic reprogramming. Meanwhile, Life Technologies is helping stem cell scientists to move away from the use of animal-derived products—a potentially serious concern in clinical stem cell applications—with their Xeno-Free Knockout Serum Replacement medium, which can be used at every stage of iPSC research from derivation to differentiation.

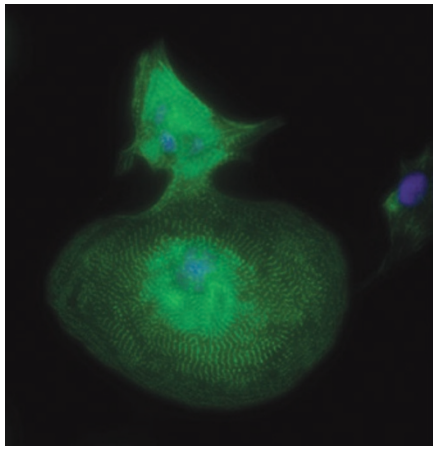
The most important and labor-intensive component of iPSC work comes after derivation of these cells: ensuring complete reprogramming and confirming pluripotency. Several assays can be used to quantify 'stemness' in promising-looking colonies, most of which measure activity of pluripotency-related genes. At the RNA level, expression can be assessed through conventional microarray analysis or via more targeted tools, such as Life Technologies' TaqMan Stem Cell Pluripotency Array, which uses quantitative PCR to measure expression of 92 different genes that are known to be active in human ESCs. Alternately, antibodies targeting several of these ESC-specific markers, such as SSEA-1 and TRA-1-60, are available from several different companies, making immunofluorescence analysis a simple option as well.

BOX 1 COMMUNITY SPIRIT

With a still-limited number of entrants into an incredibly hot research field, one might predict a fiercely competitive environment—but surprisingly, collaboration is topping the agenda for these first-round induced pluripotent cell (iPSC) companies. "It's clear to us that 99% of the science will happen outside of iPierian," says Berta Strulovici, iPierian's chief technology officer, "[and] we rely heavily on identifying new technologies from outside and having strong collaborations." Indeed, iPierian has recently engaged in high-profile disease research partnerships with Johns Hopkins and the University of Kyoto and maintains close ties with the Harvard Stem Cell Institute through the company's scientific advisory board. Meanwhile, other companies are pursuing a model of tool and technology sharing, such as CDI's CARDIOTOX consortium, launched in partnership with UK-based VivoMedica. "We're establishing a consortium of pharma customers that will provide compounds and know-how to generate a database of predictive responses when new drugs are applied to human cardiomyocytes," says Chris Kendrick-Parker, CDI's chief commercial officer.

Perhaps most ambitious is the Catalyst program being launched by Stemgent and Fate Therapeutics, which aims to develop a highly streamlined iPSC production and screening workflow that will be made openly available to any company that chooses to join their 'syndicate'—an unconventional approach that is nevertheless starting to gain momentum. "There have been mixed reviews quite honestly on the concept of everyone getting in the same room," admits Fate Therapeutics chief financial officer Scott Wolchko. "[But] every syndicate member is benefiting from the advancement of the technology that is taking place across the group, and at the end of the day, I think that's the benefit of Catalyst,… that a nascent technology platform can be developed and then shared across members."

The 'gold standard' test of pluripotency, however, is the capacity to differentiate into cells from all three germ layers: endoderm, mesoderm and ectoderm. In mice, this is typically demonstrated through chimerism experiments, in which iPSCs are implanted into early-stage embryos to determine their capacity to develop into the full range of adult tissues. Obviously, this is not an option in humans. "The only thing you can do for human iPSCs is take that cell line and put it into a teratoma assay in a mouse, and ask if the tumor tissues that form in the kidney capsule of a mouse contain all three lineages," says Chang. "That is the accepted assay, but whether it's a great assay is to be debated," he adds.



CDI has developed reliable methods for the differentiation of iPSCs into mature cardiomyocytes such as these, which are immunolabeled to reveal sarcomeric α-actinin—a key component of the muscle contractile system. Cell nuclei are stained blue with Hoechst 33342. Image courtesy of Cellular Dynamics International.

Even iPSCs capable of teratoma formation may only be 'partially reprogrammed' or may have undergone genetic damage or unnatural epigenetic modification. "If you reprogram [a somatic cell] by cell fusion or nuclear transfer, the process is done in 24–48 hours," says Zaehres. "But if you do it for weeks, as in the process of iPSC generation, you can accumulate more and more mutations." With so many open issues, quality control is likely to remain a major topic of discussion for some time to come, says Ding. "It's still under debate in the field about what is the standard that people should demonstrate with regard to pluripotency, especially for human iPSCs," he adds.

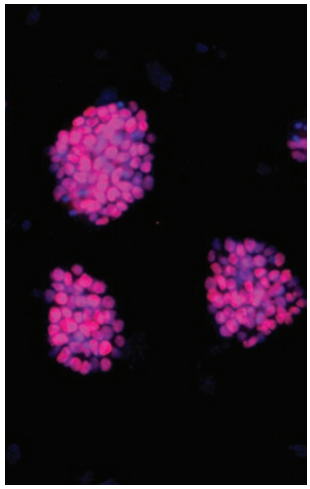
Put to the test

With direct transplantation of iPSCs fairly far over the horizon, clinical research

in this field is now focused primarily on understanding mechanisms of stem cell differentiation and applying that knowledge toward the development of efficient and reliable techniques for deriving and cultivating cell types and tissues of interest from iPSCs.

Cells collected from human biopsies are limited in quantity, vary from batch to batch and often lack the functions researchers hope to study, and iPSCs could provide limitless supplies of genetically identical cell types that would be difficult or impossible to get otherwise. CDI has already met with some early success in the use of iPSC-derived cardiomyocytes for the characterization of drug effects. "Adult heart cells coming out of a cadaver don't beat," says Chris Kendrick-Parker, CDI's chief commercial officer. "What our customers want to know is: if they use a drug known to be cardiotoxic on these cells, do the cells exhibit a cardiotoxic result? and can they compare it to *in vivo* response? And so far with the cells we're manufacturing, that's exactly what we're finding," he notes.

A unique advantage of iPSCs relative to ESCs is the potential to generate patient- and disease-specific tissues, and this is the primary focus of work now underway at iPierian. "It's really based on using iPSCs as model systems for drug discovery," says Berta Strulovici, iPierian's chief technology officer, "from learning basic biology down to performing '*in vitro* clinical trials', where you take cells from potential patients and differentiate them into relevant cells of interest and assess whether drugs that are being developed could actually cause those cells to respond appropriately." iPierian is already beginning to leverage its expertise in cell differentiation into partnerships with various academic institutions, taking on diverse neurodegenerative and metabolic diseases.



Fate Therapeutics uses a combination of proteins and small molecules to produce iPSCs for their clinical research programs. These cells are stained for expression of Nanog, a known pluripotency factor.

CDI is pursuing similar goals by exploiting the capabilities of patient-specific iPSCs for exploring pharmacogenomic variability. "We're planning on developing panels of [iPSC] lines, and one of the first is an ethnic diversity panel that looks at individuals of different races," says Kendrick-Parker, adding: "Our customers have been telling us that the ability to do *in vitro* clinical trials will have the benefit of better representing in a preclinical environment what happens in a phase I clinical trial."

Meanwhile, Fate Therapeutics is taking a somewhat different approach, guided in part by the perspective of company cofounder Ding. The company hopes to use iPSCs to model the 'niches' that support and provide instructions to adult stem cells throughout the body. "Those niches could be selectively targeted with very conventional pharmaceuticals for some kind of therapeutic benefit," explains Fate chief financial officer Scott Wolchko.

With so much left to learn about the clinical and research potential of iPSCs, the field is witnessing an encouraging amount of collaboration between scientists in both academia and industry (**Box 1**), raising hopes that rapid progress in the derivation and manipulation of these cells might help make up for some of the time lost in negotiating the minefield of hESC work.

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2. Yu, J. *et al. Science* **318**, 1917–1920 (2007).
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5. Zhou, H. *et al. Cell Stem Cell* **4**, 381–384 (2009).
6. Lin, T. *et al. Nat. Methods* **6**, 805–808 (2009).

Michael Eisenstein is a freelance science journalist based in Brooklyn, New York, USA (incandenza66@gmail.com).

SUPPLIERS GUIDE: COMPANIES OFFERING PRODUCTS AND SERVICES FOR IPSC PRODUCTION

Company	Web address
Allele Biotechnology	http://www.allelebiotech.com/
ArunA Biomedical	http://www.arunabiomedical.com/
BD Biosciences	http://www.bdbiosciences.com/
Bio-Rad	http://www.bio-rad.com/
Cellartis	http://www.cellartis.com/
Cell Biolabs	http://www.cellbiolabs.com/
Cell Line Genetics	http://www.clgenetics.com/
Cellular Dynamics	http://www.cellulardynamics.com/
CET	http://celleng-tech.com/
Cyagen Biosciences	http://www.cyagen.com/
Fate Therapeutics	http://www.fatetherapeutics.com/
Fisher Scientific	http://www.fishersci.com/
Glycosan	http://www.glycosan.com/
iPierian	http://www.ipierian.com/
Life Technologies	http://www.lifetech.com/
Millipore	http://www.millipore.com/
Miltenyi Biotec	http://www.miltenyibiotec.com/

Company	Web address
Open Biosystems	http://www.openbiosystems.com/
PAA	http://www.paa.com/
PromoCell	http://www.promocell.com/
ProteomTech	http://www.proteomtech-inc.com/
R&D Systems	http://www.rndsystems.com/
Reinnervate	http://www.reinnervate.com/
Sangamo	http://www.sangamo.com/
Sigma Aldrich	http://www.sigmaaldrich.com/
STEMCELL Technologies	http://www.stemcell.com/
Stemgent	http://www.stemgent.com/
TATAA Biocenter	http://www.tataa.com/
Thermo Scientific	http://www.thermo.com/
Transposagen	http://www.transposagen.com/
Vitro BioPharma	http://www.vitrobiopharma.com/
Vitrolife	http://www.vitrolife.com/
WiCell	http://www.wicell.org/
Zen-Bio	http://www.zen-bio.com/