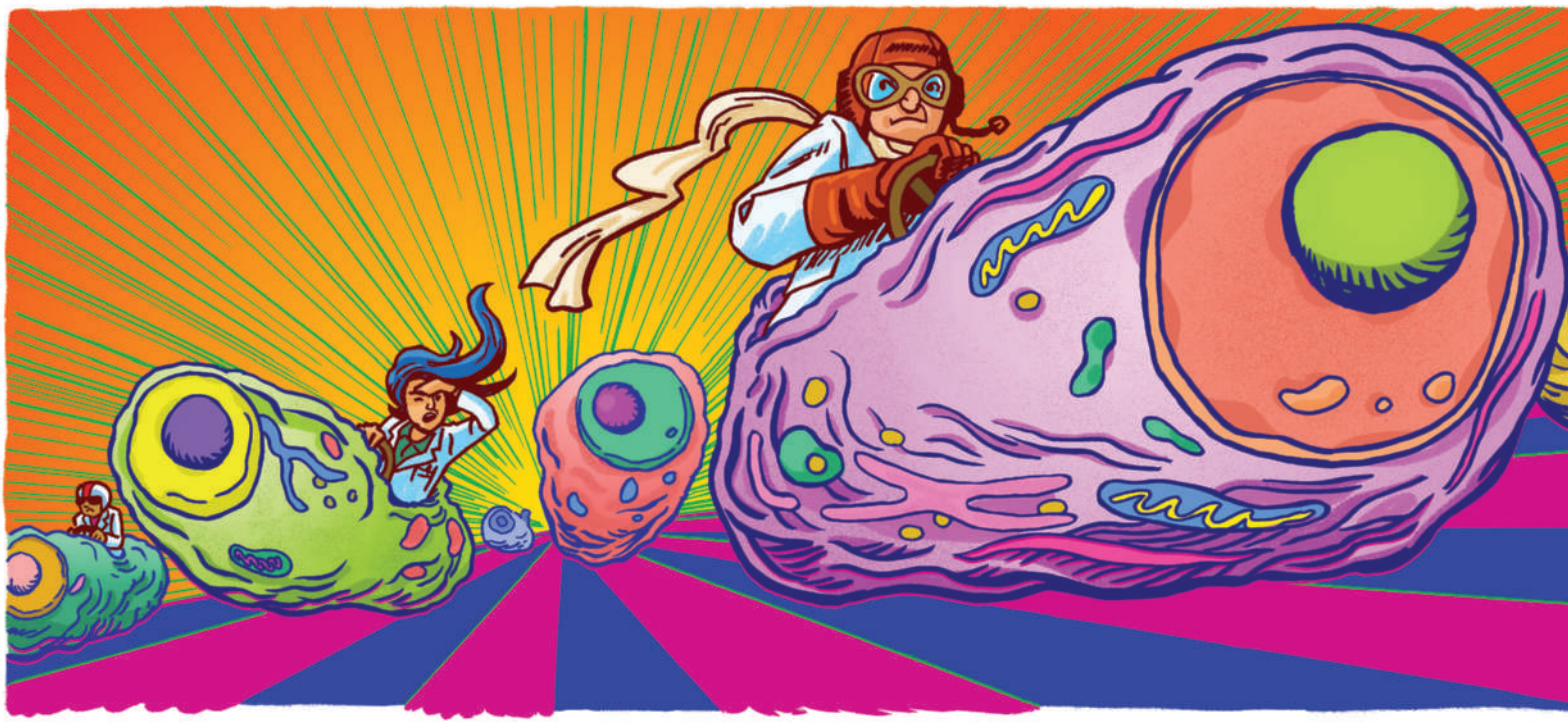


ILLUSTRATIONS BY J. RIORDAN

# FAST AND FURIOUS

The field of induced pluripotent stem cells has gone from standing start to headlong rush in less than three years. **Monya Baker** charts the course so far, and the obstacles ahead.

Back in spring 2007, Shinya Yamanaka thought he had a safe head start in a scientific race. Less than six months earlier he had demonstrated a technique that turned run-of-the-mill body cells into ones much like mouse embryonic stem cells[1]. Yamanaka's results were met with awe and scepticism. Few believed that a cell's identity was so flexible that the insertion of just four embryonic genes could reprogram it into a cell that could make virtually every body tissue.

Yamanaka knew he would have to do more to convince others that the cells were truly pluripotent: capable of becoming any cell type, including contributing to sperm or egg cells and thus another generation of animals. So on 6 June 2007, when he published an improved version of his technique showing that these 'induced pluripotent stem (iPS) cells' could actually do this[2], he hadn't expected that two other laboratories would announce that they had accomplished the same feat on the same day[3,4]. "It was less than ten months after our publication," Yamanaka recalls, "so we were very, very surprised — and we were very, very scared."

Surprise and fear are feelings that Yamanaka has become accustomed to since he founded the iPS field in mid-2006. At that time, it was just him and his lab at Kyoto University in Japan. Since then, Addgene, a company based in Cambridge, Massachusetts, has received more than 6,000 requests from in excess of 1,000 labs for the relevant reprogramming vectors that it supplies. In 2008, Harvard University, along with universities in Toronto and Kyoto, established entire facilities devoted to iPS cell studies. In 2009, researchers expect the field to move even faster and to become more competitive. In March alone, four papers in *Nature*, *Cell* and *Science* reported major refinements to the reprogramming technique[5–8].

The fervour is understandable. iPS cells promise nearly everything embryonic stem cells do — including the potential for cell therapy, drug screening and disease modelling — without most of the ethical and technical baggage. Much of the early human embryonic-stem-cell work was restricted to scientists who had access to human embryos, says Peter Andrews, a stem-cell scientist at the University of Sheffield, UK. The invention of iPS technology, he says, "opens up the area to anyone who is a competent molecular or cell biologist". Although it took 17 years from the 1981 isolation of mouse embryonic stem cells to the isolation of their human counterparts, that transition took less than six months for iPS cells. And although stem-cell researchers have yet to make patient-matched human embryonic stem cells, they have already reached an equivalent goal in the iPS cell field, making cells from patients with conditions such as diabetes, Huntington's disease and muscular dystrophy[9].

**"Every day in the lab people are worried about getting scooped."**
— Rudolf Jaenisch

Biologists are now jostling to reach the next obvious goals: iPS cells that represent a wider variety of diseases, and safer, more efficient ways to make them. "It's not healthy. It's overheated," says Rudolf Jaenisch, a leading researcher in embryonic stem cells and iPS cells at the Whitehead Institute of Biomedical Research in Cambridge, Massachusetts. "Every day in the lab people are worried about getting scooped." That means people rush to publish prematurely, he says, and are reluctant to share. "Everyone is doing very similar things, so people aren't that open to talking about papers submitted or in press."

The field risks losing sight of the big questions, says Jeanne Loring, the director of the Center for Regenerative Medicine at the Scripps Research Institute in La Jolla, California — questions such as the mechanisms by which reprogramming works and precisely what reprogrammed cells will be able to do therapeutically. "Making the cells is not the end point," says Loring. "The cells are of no value if they don't tell you something new."

When it comes to studying and treating human diseases, iPS cells are potentially far more useful than embryonic stem cells. They could eventually offer a method for taking


cells from a patient's body, treating them, and turning them into therapeutic cells that can be returned to the same individual without the risk of rejection. Researchers have already taken the iPS cells created from patients with neurodegenerative diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy and converted them into neurons[10,11]. And in mice they have taken the next step, generating blood and neural cells and using those to ameliorate mouse versions of sickle-cell anaemia and Parkinson's disease[12,13]. More immediately, the cells could be invaluable for researchers who want to study, say, brain or heart diseases and who can't collect enough tissue from biopsies or cadavers to conduct rigorous experiments. iPS cells made from patients with these diseases promise a limitless supply, and the ability to study the disease process in a dish.

## Full stem ahead

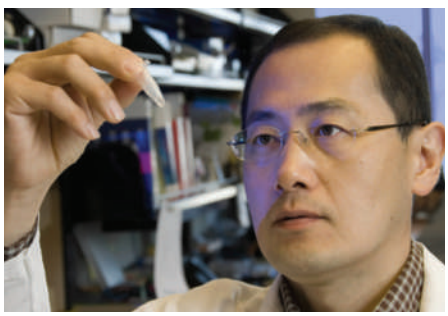
But in the first two years, researchers have been most preoccupied with improving the methods for making iPS cells. Yamanaka experimented with two dozen genes expressed in embryonic stem cells before hitting on a quartet (c-*Myc*, *Klf4*, *Oct4* and *Sox2*) capable of reprogramming adult cells when they were inserted into the genome using a virus. Although a cell dials down the activity of these genes as it assumes pluripotency, their addition nevertheless seems to make the cells less predictable and more dangerous than embryonic stem cells. Cells that are reprogrammed with the cancer gene c-*Myc* and then incorporated into mouse embryos, for example, result in animals that develop fatal tumours[1]. And Yamanaka has presented unpublished work that even mice generated from cells reprogrammed without using c-*Myc* have shorter lifespans.

The rush to develop safer, more effective techniques for reprogramming cells has often resulted in prominent publications right on the heels of each other (see 'Mile markers', overleaf). The aim has been to reprogram cells without pushing genes into the genome, where they risk causing damage. Jaenisch led one of the groups that published techniques last month for cutting out the reprogramming genes after they have finished their job[7]. Three weeks later, James Thomson and colleagues at the University of Wisconsin, Madison, reported in *Science* that they had reprogrammed human cells without requiring any genetic insertion at all[8]. They put pluripotency genes into cells using DNA rings called plasmids that did not integrate into chromosomes.

> **"The cells are of no value if they don't tell you something new."**
> — Jeanne Loring

But scientists want to do better. The worry is that reprogramming might shove cells so far from what is physiologically normal that they become pathological. The reprogramming process inhibits tumour-suppressing pathways and activates oncogenic ones, says Sheng Ding, a chemist at the Scripps Research Institute. It also disrupts a cell's processes for placing 'epigenetic' marks that control which genes are activated. "Cells are undergoing very stressful conditions," Ding says. "People don't talk about the hidden problems." Ding and many others are working to ease the transition to pluripotency with further refinements to the technique. They have found that adding drug-like molecules or starting with certain cell types can allow reprogramming with fewer types and copies of reprogramming genes as well as boosting reprogramming rates. And by the end of this year, many researchers expect to see multiple techniques for making iPS cells without adding reprogramming genes at all, instead using combinations of small molecules and proteins.

But even that won't be enough to ensure that the cells are safe for therapeutic purposes, says Thomson. Any reprogramming technique runs the risk of causing mutations or problematic epigenetic changes. "It doesn't matter whether you do it chemically or genetically, you're going to have to look at the resulting genome in excruciating detail," Thomson says.



Shinya Yamanaka made mouse iPS cells in 2006.

Yamanaka agrees. "Everyone tends to think that if you make iPS cells with fewer factors or even zero factors, with chemicals, that those iPS cells are safer, but I'm not sure about that. We really have to test each clone," he says. "Improving the derivation method is important, but I can't stress enough that how to evaluate established iPS cells is much more important."

Researchers expect the field to start shifting towards this type of evaluation. "It will be important now to compare the different methods and go with the one that works the best," says Konrad Hochedlinger of the Harvard Stem Cell Institute. What 'best' is may depend on the application. The techniques for inserting reprogramming genes are faster and technically less demanding and, for laboratories that don't have other reprogramming systems up and running, they might be a sensible choice.

## Side by side

Researchers also want to compare the iPS cells with each other and with embryonic stem cells. Embryonic stem cells are considered the gold standard. They have been studied for more than a decade, and their common origin from embryos suggests, to most scientists, that they will be less variable than iPS cells derived from different tissue types. In his recent *Cell* paper, Jaenisch characterized human iPS cells before and after the extra genes had snipped themselves out[7]. Cells that still contained extra copies of the reprogramming genes expressed 271 genes differently from embryonic stem cells; with the genes gone, that number dropped to 48. No one knows why. "There is so much anecdotal evidence saying that iPS cells don't do as well or that they are different from embryonic stem cells," says Jaenisch, "It's just unpublished." The cells could be intrinsically unique because they don't come from embryos, or they might differ from embryonic stem cells because current methods for creating iPS cells are inadequate.

Researchers have not yet agreed how to evaluate iPS cells. The most rigorous test of reprogramming involves inserting reprogrammed mouse cells into an embryo, implanting it into a surrogate mother, letting the chimaeric mice grow to adulthood, and waiting to see if the reprogrammed cells go on to make sperm or eggs that produce healthy offspring. The ability to contribute to a brand new embryo shows that the biological settings in the original cells have been reset.

Such tests are ethically unacceptable in humans, so the standard assay, borrowed from human embryonic stem cells, involves

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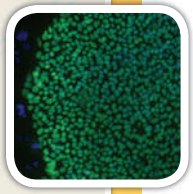
# MILE MARKERS

**AUGUST 2006**
Shinya Yamanaka uses four genes to make the first mouse induced pluripotent stem (iPS) cells[1].

**JUNE 2007**
Mouse iPS cells are shown to make all cell types[2–4].

**NOV–DEC 2007**
Human cells are induced to pluripotency[16–18].



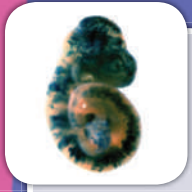
The oncogene c-*Myc* is shown to be dispensable for reprogramming[19,20].

iPS cells cure mice with sickle-cell anaemia[12].

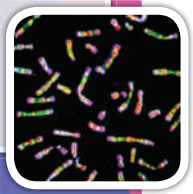
**AUGUST 2008**
Human iPS cells are made from patients with multiple diseases[9,10].

**SEPT–OCT 2008**
Two groups reprogram mouse cells without detectable DNA integration[21,22].

**DECEMBER 2008**
iPS cells from patients with neurodegenerative disease suggest that it is possible to model disease in a dish[11].



**MARCH 2009**
Researchers splice reprogramming genes out from iPS cells[5–7].



Human iPS cells are reprogrammed without genetic integration[8].

injecting human cells into an immune-compromised mouse and waiting six to eight weeks to see if the cells form a tumour called a teratoma. Naturally occurring teratomas can grow into a knot of differentiated tissues, including hair and bone, but for transplanted cells to win the iPS label, researchers just need to see a mass of differentiated cells representing all major classes of tissue. Researchers say that it is not uncommon for cells that seem fully reprogrammed in terms of appearance and surface markers to fail to form teratomas.

Some researchers think that anything worthy of the iPS cell designation should demonstrate the ability to make teratomas. "Unless we hold the field to some standard, it will muddy the literature," says George Daley at Children's Hospital Boston in Massachusetts, a leader in the field. Especially while the field is young and techniques are still being developed, he says, it is "hazardous" to say cells are iPS cells just because they express some markers typical of embryonic stem cells. "What will it mean if we call everything that has some quality of stemness an iPS cell?" Daley asks. "The term will start to lose its integrity."

**"Unless we hold the field to some standard, it will muddy the literature."**
— George Daley

## Safety first

But in some cases it may not matter if a cell line can make every cell type. iPS cells that can't make teratomas but are very good at making hepatocytes, for example, might be better for modelling liver disease and safer in the clinic. The teratoma assay is also expensive, says William Stanford of the Ontario iPS Cell Facility in Toronto. His group is generating disease-specific lines from patients at the Hospital for Sick Children in Toronto, and they already anticipate having more samples submitted for reprogramming than resources to generate cell lines. "We talked about whether we should make fewer lines and do teratoma testing on all the lines, or make more lines," he says. They decided on the latter. They will assess the pluripotency of reprogrammed cells using gene expression and *in vitro* tests of early differentiation, but further characterization will generally be left to individual laboratories that later use the cells.

Besides evaluating the iPS cells themselves, researchers also want to see rigorous, long-term evaluations of the specialized cell types generated from them, which might be used for cell therapy, drug screening, or other applications. Because obtaining homogeneous samples of differentiated cells is difficult, says Jaenisch, no one has yet published these types of evaluations. But to screen drugs or to model diseases, researchers need to be confident that, say, neurons or cardiomyocytes coaxed from iPS cells go on to age and develop disease like the cells in intact brains or hearts. And when it comes to cell therapy, they need to know that the cells are stable, and do not contain leftover iPS cells that could generate tumours, a possibility that is also being evaluated for embryonic stem cells.

Even when they have been evaluated in these ways, iPS cells will still face formidable hurdles before they reach the clinic. Regulators will need to be convinced that the risks are acceptably low and that there is a real likelihood that introduced cells will survive in the body and increase the function of a diseased brain or pancreas. It took more than a decade from the generation of the first human embryonic stem cells to the approval this January of a clinical trial of cells derived from them. Now that iPS cells can be made without genetic modification, they could make that transition much more swiftly. Yamanaka thinks that the cells will be used widely for drug screening and toxicity testing within three or four years. He hopes to see clinical trials in ten years.

Much of this work is likely to be performed by companies, and a few are already trying to corner the market in iPS cells for practical applications. John Walker, chief executive of biotech start-up iZumi Bio, in South San Francisco, California, gives little away, but says the company will focus on drug testing rather than cell therapy for now. iZumi, along with the Wisconsin Alumni Research Foundation in Madison, and others, have filed intellectual-property claims around iPS cells and the techniques for making them. With more and more methods being published, the intellectual-property situation is "more complicated than for human embryonic stem cells by an order of magnitude", says Ken Taymor, director of the Berkeley Center for Law, Business and the Economy in California.

The scientific landscape is also becoming more complex. Biologists have long assumed that one specialized cell type must be transformed back into an embryonic-like pluripotent state before it can be turned into another specialized cell. But recent work has shown that it is possible to bypass pluripotency and hop directly from one cell type to another. Doug Melton, a developmental biologist at Harvard University, did this to much acclaim in 2008 when he showed that cells in the pancreas could take on the appearance and function of insulin-producing β-cells if extra copies of

FROM BOTTOM TO TOP: CNRI/SPL; REF. 5; ISM/SPL; M. KULYK/SPL; ISM/SPL; PASIEKA/SPL; REF. 20; S. YAMANAKA, KYOTO UNIV.; REF. 4; MEDICIMAGE/PHOTOLIBRARY.COM

pancreatic genes are inserted into them[14].

Whether reprogramming proceeds 'backwards' or 'sideways', scientists want to understand how it occurs. For many established scientists, this is the question that brought them into the iPS-cell field in the first place. Yamanaka says he would not have attempted his initial reprogramming experiments were it not for the cloning of frogs by nuclear transfer in the 1950s or the cloning of Dolly the sheep in 1996. Before that, some thought that genes were irreversibly deactivated or perhaps even excised as cells progressed through development. Dolly — cloned from an adult cell — showed that the genes remained intact and amenable to rebooting, even in specialized mammalian cells.

**"It's all technology, technology, technology. Now we are coming to the interesting questions."**
— Rudolf Jaenisch

## Rough guide

Researchers understand the general outlines of reprogramming. Cells loosen the tangles of DNA and protein, known as chromatin, and rearrange epigenetic marks so that the genes active in specialized cells are silenced, and those active in embryonic stem cells are turned on. They recruit an army of proteins to shift the cell machinery from one state to another. How and when all these steps occur is, despite intense study, still being worked out — and it is a question that many researchers hope that the iPS field will focus on as it matures. With iPS cells "you can ask how reprogramming really works", says Hochedlinger, "This was a question that was raised 50 years ago. We have no clue."

iPS cells don't make the problem easy, though. For one thing, it is difficult to isolate the right cells: typically, less than 1 in 1,000 cells is successfully reprogrammed in the production of iPS cells. Some cells remain trapped in differentiated states even if pluripotency genes are active[15]. "The problem is that we don't have the intermediate states," says Kathrin Plath, a cell biologist at the University of California, Los Angeles. Using gene expression and cell morphology, Plath is studying a subset of cells that seem to get stuck on the way to full reprogramming. "Partially reprogrammed cells seem to be very similar no matter how you get them," she says, "but who knows if they are true intermediates of the actual reprogramming process or off on a side track?"

Understanding the reprogramming process is not just an academic exercise. Knowledge about the various states of a cell, and how cells move from one state to another, could help researchers refine their techniques for driving cells through those transitions safely, and making the cell types they want for therapies.

Researchers who have seen other biological fields, such as recombinant DNA and RNA interference, go through a similar breathless period after their inception, predict that the frantic pace and competitiveness are likely to wane. The rush to optimize the reprogramming techniques will pass, predicts Martin Pera, director of the Institute for Stem Cell and Regenerative Medicine at the University of Southern California, Los Angeles, and scientists will branch out into particular types of disease or more fundamental questions. "Activity in the field will diversify," he says, "and the field will become more collaborative."

Collaborative or not, the iPS race is heading into a new, and perhaps more intellectually rewarding, leg. Until this point, "it's all technology, technology, technology", says Jaenisch. "Now we are coming to the interesting questions. And the challenging questions will be the biological questions." ■

**Monya Baker is the editor of *Nature Reports Stem Cells*.**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