



TAKE ONE ADULT SKIN CELL:

Add four genes borne by viruses // watch as the cell reverts to infancy // steer it toward a **different purpose**.

The Other Stem Cells

■ BY RACHAEL MOELLER GORMAN // ILLUSTRATION BY MIRKO ILIĆ

When a garter snake or the occasional bird attacks a newt, the predator often is able to snatch a limb or tail before the newt can slip away. A loss like that would be devastating to most creatures, but to this amphibian, it's manageable. Within a day, the injury begins to heal; within a month, skin cells at the wound site actually start changing identity, moving backward in developmental time. The progenitor cells that those skin cells become are able to turn into all of the different cells the newt needs to create a leg. Just 10 weeks after the attack, five toes have pushed their way out of the growing mass of skin, cartilage and muscle, and, finally, the new limb emerges.

More complex species could never pull off the newt's trick. "Development in mammals is a one-way road," says Konrad Hochedlinger, a stem cell biologist at the Massachusetts General Hospital and Harvard University. "Once a cell becomes a skin cell, it remains a skin cell for the rest of your life. You can't grow a liver, say, on your arm. It just doesn't happen."

Yet in 2006, Shinya Yamanaka at Kyoto University in Japan reported a "crazy" experiment (Hochedlinger's word) to show that mouse skin cells in a petri dish, aided by four genes and viruses that inserted the genes into the cells' DNA, could achieve something like the newt's developmental reversal, becoming just about any cell in the mouse's body. The new cells, which Yamanaka called induced pluripotent stem (iPS) cells, looked and behaved like embryonic stem cells, which are prized for their ability to transform themselves into almost

any kind of tissue and, perhaps, someday cure disease—a more distinct possibility now that President Barack Obama has loosened restrictions on stem cell research.

Harvesting embryonic cells, however, is controversial because it results in the destruction of an embryo. The hope, in the wake of Yamanaka's discovery, was that iPS cells could be a controversy-free alternative, conferring on humans a newtlike ability to regenerate lost or diseased tissue. Stem cells of either sort might be able to create healthy neurons for a patient with Parkinson's disease, for example, or replace damaged or diseased cells of almost any type, promoting recovery from a wide range of maladies. IPS cells have already been used to treat sickle cell anemia in mice and Parkinson's disease in rats. And iPS cells may have a particular advantage: Taking a person's own skin cells, say, making them pluripotent, and then using those cells to grow whatever kind of tissue is needed could eliminate the use of debilitating immunosuppressive drugs, which are required when transplanting cells or tissue from a donor.

There were problems with Yamanaka's initial technique for creating iPS cells, including an elevated risk of cancer and a very low efficiency rate, with only one in about 10,000 cells that received the four genes becoming an iPS cell. But a flurry of research findings published during the past few months indicates that several new approaches may get around those issues. One study even suggests it's possible to reprogram cells inside an animal's body, in this case changing exocrine cells in the pancreas into insulin-producing beta cells that might be used in diabetes treatments. "This is a completely unexpected

ground shift in the whole field of cell biology,” says Clive Svendsen, a stem cell biologist at the University of Wisconsin, Madison. “Every time I wake up in the morning, I just cannot believe this has happened.”

Mammalian development normally proceeds in a steady-fast forward march. A sperm fertilizes an egg, the egg divides once, those cells divide and the process continues until a ball of cells forms. About four days after fertilization, that ball has developed into a blastocyst, and many of the cells within it are embryonic stem cells. (Researchers can extract those cells, typically from unused embryos created during in vitro fertilization, but that process destroys the blastocyst.) The DNA within ES cells is the same as that found in adult heart, skin and eye cells, but it exists in a very open form. ES cells are pluripotent—they can become any cell in the body—because all of their genes have the potential to be turned on.

That’s very different from adult cells. In the process of becoming a liver cell, for example, many of a cell’s genes are turned off or disabled. The DNA of those genes is folded tightly, and chemicals called methyl groups attach themselves to the genes, preventing the transcription of genetic code that enables the proteins associated with the genes to form. With whole sections of DNA turned off, a liver cell can make only the proteins needed for the liver to function.

These gene shutdowns were once thought to be permanent. But it turns out that a process called somatic cell nuclear transfer (SCNT) can sometimes reactivate genes. During the 1950s and 1960s, researchers working with frogs developed techniques to extract the nucleus of a differentiated adult cell and stick it into an enucleated, unfertilized egg. Something about the environment of the egg again turned on all of the genes in what had been a differentiated nucleus, reprogramming the adult DNA to its embryonic state, and the newly pluripotent cell was able to grow into a tadpole. In 1996 researchers

Frozen but hardly halted, the lines of reprogrammed stem cells in the Massachusetts General Hospital laboratory of biologist Konrad Hochedlinger are preserved in liquid nitrogen at -170°C .

in Scotland used SCNT to transplant the nucleus of an adult sheep cell into an enucleated sheep egg, producing Dolly, the first cloned mammal. Other animal clones followed.

Cloning was very difficult, however. Few cell transplants worked, and fewer still yielded healthy adult animals. Researchers assumed that whatever happened in the egg to return an adult cell to its pluripotent state must be exceedingly complex.

Then came Yamanaka’s study, published in the journal *Cell* in August 2006. “To everyone’s jaw-dropping surprise, you needed only four genes,” says stem cell biologist David Scadden, director of the Center for Regenerative Medicine at the MGH and co-director of the Harvard Stem Cell Institute. To find those genes, Yamanaka had compared all the genes (the genome) of an embryonic stem cell with those of an adult cell to find genes that were turned on in the ES cell but turned off in the adult cell. Those, along with genes already known to be important in embryonic stem cells, totaled 24, and, using a virus to carry them, he inserted the genes into skin cells taken from the tail of a mouse. Some of the new cells, as they divided and grew, started to change. They shrank and clumped into balls of cells, reversing their developmental course and eventually becoming surprisingly similar to embryonic stem cells.

Using 24 factors made the process very involved, so Yamanaka set out to learn whether all of the genes were needed.



GREGORY HEISLER FOR PROTO

He repeated the experiment with combinations of fewer and fewer genes until he determined that he could create iPS cells with just four genes that code for four transcription factors (a type of protein that turns genes on or off in a cell): Oct3/4, Sox2, c-Myc and Klf4. “They clearly act as some master regulators and kick-start a process that does a lot of things to the cell that eventually gives rise to pluripotent cells,” says Hochedlinger. “But how it works, we don’t know.”

In late 2007, James Thomson at the University of Wisconsin, Madison; In-Hyun Park and George Daley at Harvard University; and Yamanaka each reported creating human iPS cells, using either the same four genes that had worked in mice or, in the case of Thomson, two of those genes, Oct3/4 and Sox2, plus two different ones, Nanog and Lin28.

Throughout these experiments, researchers noticed that only one in about 10,000 skin cells receiving the four genes ever becomes an iPS cell—an inefficiency that could undercut

“This is a completely unexpected ground shift in biology. Every time I wake up, I just cannot believe this has happened.”

the potential therapeutic value of the cells. An even bigger obstacle is that two of the four factors, c-Myc and Klf4, are oncogenes—they cause cancer. And when viruses containing the four genes insert themselves into the genomes of skin cells, they can activate innate cancer genes and cause tumors.

Six of One... //

When President George W. Bush banned federal funding for research on embryonic stem (ES) cell lines created after Aug. 9, 2001, stem cell research was significantly hampered. Of the 71 preexisting ES cell lines (genetically identical, immortal cultures of cells) for which Bush did permit funding, only 21 ended up being available to researchers (for example, some lines suffer when frozen for shipping). This is too few for a field that needs a wide array of genetically diverse material to support its mission to cure disease. Daunted, many researchers shied away from working with ES cells. Now that is likely to change, given President Barack Obama’s reversal of the ban. A higher number of healthy ES lines will allow better study of human development, but iPS cells will not fall by the wayside. Here’s why both types are important.

	EMBRYONIC STEM (ES) CELLS	INDUCED PLURIPOTENT STEM (IPS) CELLS
POTENTIAL	→ Replacing cells crippled or killed by disease, including heart disease, Alzheimer’s disease and type 2 diabetes	
SOURCE	→ Human embryos, especially embryos created during in vitro fertilization (IVF) that do not end up being implanted	→ Adult cells, such as skin or the root of a hair
PROS	→ Pluripotent (can become any cell in the body) → Regenerate continuously (important because large numbers of cells are needed to replace diseased tissue)	
	→ Considered the gold standard; have been studied eight years longer than iPS cells	→ Relatively easy to make → Can be made genetically identical to the patient (sidestepping possible immune system attack)
CONS	→ Unused IVF embryos are weaker than embryos that have been implanted, which could affect the viability of their cells → Too few IVF embryos are available to supply the growing demand → ES cells from IVF embryos would require immunosuppressive therapies so that the tissue wouldn’t be attacked by patients’ immune systems	→ Clinical and scientific utility have yet to be fully confirmed → Two of the four genes used to turn back the cells’ developmental clock might cause cancer, as might some of the viruses that carry the genes (though research is beginning to get around these problems) → Low efficiency—for every iPS cell created from a skin cell, several thousands of other skin cells in the experiment remain unconverted
MORAL OBJECTIONS	→ Human embryo destroyed while extracting the cells	→ None
STATUS OF CLINICAL RESEARCH	→ Recent approval of a clinical trial to examine the safety of inserting ES cells into spinal cords of injured patients	→ Researchers have treated disease in animals using iPS cells, but human trials are years away



Doug Melton, co-director of the Harvard Stem Cell Institute, has rewritten the formulas for returning adult cells to an earlier, more fruitful stage of development.

Dozens of research laboratories have been working to fix those problems. Hochedlinger has focused on the use of viruses to insert genes into cells, and has devised a way to produce iPS cells that only temporarily introduces reprogramming genes into the cells. He accomplished that with the help of a virus different from the retroviruses used initially: an adenovirus that brings the genes into a cell's nuclear area but doesn't become part of the cell's DNA; it disappears after it expresses the reprogramming genes.

More recently researchers have transformed mouse and human cells into iPS cells without using viruses at all. Two reports show that the four iPS genes can be inserted into the DNA of a skin cell and later excised, leaving no trace. Researchers did this by using a transposon (a piece of DNA that can jump around the genome), called piggyBac, that can be cut from the skin cell's DNA with an enzyme. Another report has shown that even when viruses are used, they can be cut out, which eliminates the cancer risk they pose.

Meanwhile, Doug Melton, the other director of the Harvard Stem Cell Institute, has tackled the oncogene issue. He decided to see whether it was possible to eliminate those two genes entirely by using a drug—valproic acid—to aid in the transformation of the skin cells. Valproic acid is an HDAC (histone deacetylase) inhibitor that loosens up DNA from its tightly packed resting state and keeps it open and ready to transcribe genes; it might be able to replace some of the reprogramming genes because a function of one of the oncogenes, *c-Myc*, is to

induce histone acetylation—the opening up of the DNA. Valproic acid reduces histone deacetylation, in effect promoting acetylation, as the oncogene does. That allows the two other genes, *Sox2* and *Oct3/4*, to bind more easily to the DNA and turn on genes that return the cell to a pluripotent state.

Melton and Danwei Huangfu, the lead researcher on the study, first used valproic acid to replace just *c-Myc*. That combination—of just three genes, *Oct3/4*, *Sox2* and *Klf4*, plus valproic acid—not only produced iPS cells but also increased the efficiency of the procedure a hundredfold. The scientists then tried removing the second oncogene, *Klf4*, inserting only *Oct3/4* and *Sox2* along with the valproic acid. That worked, though efficiency plummeted. A major advantage of using valproic acid is that it's already approved as an epilepsy drug by the Food and Drug Administration, so if it were ever to be used in humans, it wouldn't have to go through the same long, expensive approval process that a new compound would require.

Even if researchers perfect their techniques for creating iPS cells, widespread use in humans may still be years away. One intractable problem is the formation of teratomas, cancers that both ES and iPS cells generate even without oncogenes. But a different application for iPS cells is on the horizon: They can reveal for the first time what happens at the molecular level as a disease unfolds. Normally, scientists see only the end result—a heart attack, diabetes or the memory loss of Alzheimer's disease. But now it may be possible to

No one yet knows what's actually going on in iPS cells when they're reprogrammed, and cancer is still a very real problem among rodents that get the cells.

take cells from someone with a neurological disease, for example, make them into iPS cells and then, treating them with chemicals and proteins that encourage the cells to differentiate, coax them into becoming neurons. But because the new cells still have the genes that caused the disease in the first place, they are likely to get sick as well.

"You can replay a disease over and over in a petri dish," says Svendsen of the University of Wisconsin, "observing changes at the cellular level that occur long before symptoms appear." Researchers can also test drugs that might slow or halt the disease. "During the next 10 years," says Svendsen, "we're going to see a trend away from using animals for disease modeling and using human cells with iPS technology instead."

A research group at Harvard (spearheaded by Daley and including Hochedlinger's group) has already created lines of iPS cells to study 10 diseases, housing the lines at the MGH for other scientists to study. Svendsen, meanwhile, produced iPS cells using a skin sample from a child who had spinal muscular atrophy (SMA) and generated motor neurons from the iPS cells. Then he compared them with neurons made from iPS cells that originated from a healthy donor. The child's new neurons degenerated while the healthy donor's survived, showing that scientists can watch a disease progress in the laboratory dish. The next step will be to try to cure the SMA in the dish by replacing defective genes or screening for an effective drug.

took just three days instead of the three weeks needed to produce an iPS cell. In people with diabetes, beta cells don't function well, but Melton and his team were able to use the new beta cells to improve the glucose state of diabetic mice.

The potential for that approach remains to be established. And indeed all the research involving iPS cells is still at an early stage. No one yet knows what's actually going on in cells when they're reprogrammed, and cancer is still a very real problem, with most rodents that get iPS cells developing the disease. What's more, iPS cells are not perfect models for every disease. "They are good for developmental disorders such as SMA," says Svendsen, but scientists don't yet know if they will work as well with diseases that develop over many decades. "Alzheimer's disease doesn't strike most people until they're at least in their seventies, so you might have to leave the cells in the dish for 70 years until they show any symptoms," he says. "That's a little longer than my NIH grant."

Moreover, research to be published soon might show small but perhaps crucial differences between induced pluripotent stem cells and the embryonic variety that could affect potential applications. Yet such issues have done little to dampen scientists' enthusiasm. "Certain boundaries that we thought were there really aren't," says Hochedlinger. "And it may be possible to overcome problems we thought could never be solved." ■

→ DOSSIER

1. "Nuclear Reprogramming in Cells," by J.B. Gurdon and D.A. Melton, *Science*, Dec. 19, 2008. A comprehensive history of cell reprogramming and its potential, from frogs to Dolly to iPS cells.
2. "In Vivo Reprogramming of Adult Pancreatic Exocrine Cells to Beta-cells," by Qiao Zhou et al., *Nature*, Oct. 2, 2008. In the latest stem cell breakthrough, Doug Melton and his co-authors describe turning one type of adult pancreatic cell into another, inside a living mouse.
3. "Induction of Pluripotent Stem Cells From Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors," by Kazutoshi Takahashi and Shinya Yamanaka, *Cell*, Aug. 25, 2006. Revolutionizing stem cell research, Japanese scientists report creating the first iPS cell, turning a mouse skin cell backward in developmental time.

The process that Svendsen and other researchers are using—harvesting a differentiated adult cell, returning it to a pluripotent state in the lab, then sending it down the path to specialization—has achieved remarkable results. But other scientists have been exploring whether a shortcut might be possible. What if the transformation could happen in the body? In a study published in October 2008, Melton showed that it was possible to take an exocrine cell in the pancreas of a live mouse and turn it into an insulin-producing beta cell without first going back to an undifferentiated iPS state. He added only three transcription factors, and the process